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THE DISTRIBUTION AND PROPERTIES OF RENAL KININOGENASE

by

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A THESIS

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ABSTRACT

The literature pertaining to the properties and functional significance of hypotensive substances in the kidney is reviewed. The kinins and kinin-forming enzymes are discussed in a general context, and emphasis placed upon recent investigations concerning renal kininogenase.

Results

1) The ability of renal extracts to liberate kinins from a kininogen substrate was used to determine the tissue concentrations of renal kininogenase.

2) Acidification of renal extracts was found to inhibit kininase activity, this procedure facilitating the quantitative biological assay of generated kinins.

3) Kinin-forming enzymes were detected in the kidneys of several mammalian species.

4) The results of this study concur with published descriptions of the properties and localization of rat renal kininogenase.

5) Kininogenase was primarily found in the renal cortex of the dog and rat.

6) An attempt was made to ascertain a more specific intra-cortical localization of this enzyme. A technique involving the isolation of glomeruli from the dog renal cortex revealed relatively high levels of activity in this tissue. However, kininogenase was also detected in the other tissues of the renal cortex.

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7) Preliminary experiments on the production of unilateral renal artery constriction in the rat, demonstrated a significant reduction in the kininogenase concentration of the damaged kidney. It has been reported that reduced excretion of urinary kallikrein occurs under similar circumstances.

Conclusions

The role of kininogenase in the kidney remains obscure. The enzyme has been demonstrated in both the glomerular and tubular tissues of the dog renal cortex. Since tubular tissue constitutes the major bulk of the cortex, it is concluded that kininogenase is primarily associated with the tubular system. This conclusion is in agreement with results derived from other studies.

A relationship between renal kininogenase and the antihypertensive function of the kidney has not been established. The presence of kininogenase in the glomerular tissues suggests a role of intra-renal kinin release in the control of the renal vasculature. Similarities between the renin-angiotensin system and the kininogenase-kinin system are emphasized. It is concluded that the rapid destruction of kinins in the circulation prevents the kininogenase-kinin system from exerting a systemmic antihypertensive function. However, intra-renal kinin release may suppress the activity of the renin-angiotensin system by producing arteriolar vasodilatation.

Renal kininogenase and urinary kallikrein exhibit many similarities and it is concluded that urinary kallikrein is a "true" secretion of the kidney.

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INTRODUCTION

"The discovery in an artificial extract from an organ or tissue, of a substance which on artificial injection produces a pharmacodynamic effect, provides only a first item of presumptive evidence in support of a theory that the action of this substance plays a part in normal physiology. Much more evidence is required before we can attribute clearly defined functions to such a substance, as we now can do in the case of histamine and acetylcholine. But even where this is already possible, we have no evidence to justify the assumption that the substance comes naturally into the body, in the free condition in which we isolate and identify it in the laboratory after various unnatural chemical procedures."
(Sir Henry Dale, 1933)

A relationship between the kidney and the pathogenesis of arterial hypertension was intimated by Richard Bright in 1836, when he wrote "that the hypertrophy of the heart seems in some degree, to have kept pace with the advance of disease in the kidneys." The extraction of a hypertensive substance called "renin" from the rabbit renal cortex provided the first evidence of a causative factor (Tigerstedt and Bergman, 1898).

The renin-angiotensin system is now generally considered to be responsible for the initiation of renal hypertension, however, evidence has accumulated to suggest

the presence of an antihypertensive renal function. This evidence will be presented, and the vasoactive substances that may be involved are described. The renal kininogenase-kinin system is discussed within this context to promote further investigation.

Since more is known about the general properties of kininogenases derived from other sources, a separate section (APPENDIX) concerning this basic information is provided.

1. The Antihypertensive Function of Renal Tissue

The subject has been reviewed by Page and McCubbin (1968); Lee (1966, 1967); Lee et al., (1971) and Muirhead et al., (1966 a). The evidence is based upon three experimental models.

a) Unilateral renal artery constriction

Goldblatt (1937) first described the production of experimental hypertension by unilateral renal artery constriction. Blalock and Levy (1937) increased the incidence and severity of the disorder by removing the contralateral normal kidney. This finding, linked with the observation of Rodbard and Katz (1939) that the severity of the condition depends upon the ratio of ischaemic to normal tissue, was the first indication of a protective renal function. Removal of the contralateral normal kidney has become a standard procedure in the production of hypertension by unilateral renal ischaemia.

b) Renoprival hypertension

It has been demonstrated by many authors that bilateral nephrectomy leads to a salt dependent hypertension (Braun-Menendez and von Euler, 1947). Grollman, Muirhead and Vanatta (1949) maintained nephrectomised dogs for as long as 16 days with the aid of an artificial kidney. The maintenance of dogs for more prolonged periods was facilitated by intermittent peritoneal lavage (Grollman, Turner and McLean, 1951). Dogs maintained in this manner exhibited hypertension, cardiac hypertrophy and atherosclerotic lesions, however, the blood volume, cardiac output and venous pressure were normal. Similar results were obtained by Kolff and Page (1954 a) in rats.

Transplantation of a pair of normal kidneys into the necks of ten nephrectomised dogs, alleviated renoprival hypertension in nine cases, whereas the transplantation of a pair of hind legs did not provide this protective effect (Kolff and Page, 1954 b).

Grollman, Muirhead and Vanatta (1949) implanted ureters into the vena cava of dogs, producing the same degree of uraemia and loss of excretory function as in total nephrectomy. Renoprival hypertension did not develop, indicating that the protective role of kidney tissue was not based upon an excretory function. Transplantation of fragments of whole kidney, or renal medulla into the

peritoneum or lungs afforded similar protection (Muirhead, Stirman and Jones, 1960). Renal transplantation in man has been shown to alleviate renoprival hypertension (Merrill et al., 1956; Ducrot et al., 1966).

Total nephrectomy leads to a complex physiological state and controversy exists as to whether renoprival hypertension is a consequence of altered vascular reactivity due to electrolyte imbalance, or the loss of a specific antihypertensive function.

c) Renovascular hypertension

The grafting of normal renal tissue into hypertensive rats reduces the blood pressure. If the grafted kidney is perfused "in situ" at low pressure, it fails to exert an antihypertensive effect, whereas when perfused at high pressure it normalizes the blood pressure, (Gomez, Hoobler and Blaquier, 1960; Tobain, Schonning and Seefeldt, 1964; Hickler et al., 1966). As a consequence of these findings, Skinner, McCubbin and Page (1964) postulate that perfusion pressure regulates the secretion of hypertensive and antihypertensive substances.

2. Vasodepressor Substances in the Kidney

a) Non-specific agents

Early attempts to isolate renin from simple renal extracts were complicated by the presence of hypotensive impurities. Many authors report mixed pressor and depressor responses on the administration of extracts to experimental

animals (Vincent and Sheen, 1903; Shaw, 1906; Harrison, Blalock and Mason, 1936; Prinzmetal and Freidman, 1936; Pickering and Prinzmetal, 1936; Williams, Harrison and Mason, 1938).

Attempts were made to remove the hypotensive component; Landis, Montgomery and Sparkman (1938) finding that by heating saline extracts at 55° C, some of the protein was precipitated, the removal of which led to a more consistent pressor effect. It was concluded that the hypotensive component was a toxic substance produced by the action of bacteria and tissue autolysis.

The first intensive investigation of renal extracts for hypotensive substances was made by Grollman, Williams and Harrison (1940 a and b) who described the extraction of a water soluble, dialyzable agent from hog kidneys. The extract, when given by mouth or vein to hypertensive rats and a few hypertensive patients, produced a depression of arterial pressure over a period of one or two days. It is of particular interest that the extract reportedly had a negligible effect upon normal animals. As the extract became purified, it became more water soluble (Grollman, 1947, 1966) and was considered to be a peptide (Hamilton and Grollman, 1958) although evidence to substantiate this claim was not provided. Sokabe and Grollman (1962) localised the active agent to the renal cortex of the dog.

Page et al., (1940, 1941 a and b) prepared saline extracts of hog kidneys, which upon intravenous or subcutaneous

injection led to a depressor effect in hypertensive animals. The active principle, although similar in actions to the extract prepared by Grollman, differed in that it was non-dialyzable and in high doses produced shock, fever and the general signs of anaphylaxis. The specificity of the preparation was questioned by Page, Taylor and Corcoran (1951) when it was discovered that non-specific pyrogens were also partially effective.

Schales, Stead and Warren (1942) emphasized the probability that non-specific substances were responsible for the depressor effects of crude renal extracts, a criticism that is still valid because purification and pharmacological identification were never successfully completed. Gordon (1959) demonstrated that crude saline extracts of rabbit kidney contain significant concentrations of the adenosine nucleotides AMP, ADP and ATP. A mixture of these nucleotides, equal in concentration to that present in the original extracts was sufficient to induce the observed vasodepressor activity. This study is important because it points out the pitfalls inherent in the use of crude renal extracts and the blood pressure assay technique. It is clear that many substances including pyrogens can produce a hypotensive response on injection, emphasizing the need for biochemical and pharmacological identification.

b) <u>The renomedullary lipids</u>

The prevention of canine renoprival hypertension by the presence of renal medullary tissue (Muirhead, Stirman

and Jones 1960), led to the investigation of crude saline extracts of the canine renal medulla. They were found to be free of toxicity when given intravenously, and not only prevented the occurrence of renoprival hypertension, but also reduced the severity of the condition in cases where the hypertension had become established (Muirhead, Jones and Stirman 1960). The active principle was determined to be a neutral lipid, extractable in ethanol, dialyzable and separated by Sephadex G-25 as a relatively small molecule with a molecular weight less than 1,000 (Muirhead, Hinman and Daniels, 1963).

i. Neutral lipids

The neutral lipid isolated by Muirhead et al., (antihypertensive neutral renomedullary lipid, ANRL) has been shown to lower the blood pressure of acute and chronically hypertensive dogs, rabbits and rats, by oral as well as intravenous administration. It required an average of 12 days for the pressure to reach its lowest levels, thereafter the pressure remained depressed for as long as adminstration was continued. When the treatment was stopped, an average of 36 days was required for the pressure to return to the pretreatment hypertensive levels. It is particularly interesting that ANRL did not evoke a depressor effect in normal animals (Muirhead, et al., 1966 b).

ii. Basic lipids

Milliez et al., (1961) found that acetone extracts of dog and rabbit kidneys lowered the arterial pressure of hypertensive but not normal rabbits. The characteristics

of the active principle suggested a lipid with basic properties (Milliez et al., 1966).

iii. Acidic lipids, the prostaglandins

Lee et al., (1963) and Hickler et al., (1964) described the preparation of an extract of rabbit renal medulla containing an acidic lipid, which differed from that of Muirhead et al., by producing an immediate and sustained lowering of blood pressure when injected into vagotomised, pentolinium treated, normotensive rats. The crude extract was called "Medullin". It was reported by Lee et al., (1965) that three hydroxylated fatty acids of the prostaglandin type were present, identified as $PGF_{2\alpha}$, PGE_2, and PGA_2. The rabbit renal medulla was the primary tissue for study, as it contains a higher concentration of prostaglandins than any other source, with the exception of the human and sheep seminal fluid.

Daniels (1971) summarises present knowledge, reporting that few conclusions are possible concerning the distribution of the primary prostaglandins in the kidney, except that PGE_2 and $PGF_{2\alpha}$ predominate. This finding is particularly significant because PGE_2 is hypotensive, whereas $PGF_{2\alpha}$ is primarily a pressor agent.

The localization of prostaglandin synthesis in the medulla is uncertain, Muirhead et al., (1972) suggest the interstitial cells, whereas Janszen and Nugteren (1971) propose the collecting tubules.

It would be premature to propose a physiological role for the renomedullary prostaglandins. However, Lee et al., (1971) postulate that deficiency of renal prostaglandins may lead to the genesis of hypertension. They propose that prostaglandins in the venous blood or lymph draining from the kidney exert a peripheral antihypertensive effect. A reduction in the output of these substances through kidney damage or genetic factors would then lead to a preponderance of pressor mechanisms. Alpert and Hickler (1971) provide evidence from several investigations which suggest that, contrary to this model, prostaglandins are elevated during renal hypertension.

Lee et al., (1971) find that infusion of PGA and PGE compounds produce a shift in blood flow from the medulla to the cortex and hence, if the prostaglandins were to become deficient, a degree of cortical ischaemia would be expected, this activating pressor mechanisms and leading to hypertension. This latter finding is also subject to conjecture (Johnson et al., 1971), others observing that prostaglandin infusion preferentially increases non-cortical blood flow.

It is clear that the hypothesis proposed by Lee and his co-workers must be viewed with caution until these contradictory findings are clarified.

iv. Phospholipids that inhibit renin

It has been possible to purify a phospholipid renin inhibitor from dog kidneys (Sen, Smeby and Bumpus, 1967).

It inhibited the response to injected renin in the bilaterally nephrectomised rat, and reduced the blood pressure of rats with acute and chronic renal hypertension. The phospholipid had no effect on the blood pressure of normal rats.

The active inhibitor is derived from phospholipids by a phospholipase hydrolase. The structure of the pre-inhibitor was found to be a phosphatidylethanolamide, esterified with a high proportion of polyunsaturated fatty acids including arachidonate (Pfeiffer et al., 1971; Rakhit 1971). The relationship between this substance and the prostaglandins is not known, although the presence of arachidonate in the former suggests a common metabolic origin at the precursor level.

3. Renal and Urinary Kininogenase

The discovery of the kallikrein-kinin system may be traced to experiments performed by Abelous and Bardier (1908) when they noted the hypotensive properties of normal human urine. The active substance, which they called "urohypotensine" was water soluble, non-dialyzable, destroyed by boiling and precipitated by both alcohol and saturated ammonium sulphate. Pribram and Herrneiser (1920) and Migay and Petroff (1923) confirmed the hypotensive properties of urine, noting that pancreatic juice exerted a similar effect.

Frey (1926) named the active agent "F-stoff", it was found in the blood, glands and exocrine secretions of several mammalian species, and was considered to be a

circulating hormone, after it was shown that pancreatectomy led to a fall in the urinary excretion of the substance. Hence, it was re-named "kallikrein" after the Greek word "kallikreas" meaning pancreas (Frey, Kraut and Schultz, 1930). This terminology was subsequently found to be inaccurate, pancreatectomy did not alter the urinary excretion of kallikrein and the previous findings were refuted (Frey and Werle, 1933).

The name "kallikrein" is a misnomer, which has remained in the literature to designate a group of enzymes with similar properties and biological effects. Kallikreins derived from different tissues are not identical and a common pancreatic origin is no longer a tenable concept.

The enzymic nature of kallikrein was demonstrated by Werle (1936) after the incubation of human urinary kallikrein with plasma. The incubate contained a thermostable substance of low molecular weight that proved to be a potent stimulant to the isolated guinea-pig ileum. The substance was named "darmkontrahierende substanz" or substance DK (Werle, 1937), and later re-named "kallidin" (Werle and Berek, 1948).

Quite independently, Rocha E Silva was studying the cardiovascular effects of snake venoms and trypsin. It was found that antihistamines and atropine were very limited in their therapeutic efficacy against the shock reactions induced, and it became clear that substances other than histamine and acetylcholine were involved. Investigation

of the venom of B.jararaca, a strongly proteolytic venom of a common Brazilian snake, led to the discovery of a new substance called "bradykinin" (Rocha E Silva and Beraldo, 1949; for review, Rocha E Silva, 1970). When the venom was added to plasma, a potent stimulant to the guinea-pig ileum was liberated, which produced a characteristic "slow" contraction unlike that produced by acetylcholine or histamine, hence the name bradykinin (bradys-slow, kinean-movement).

Kallidin and bradykinin were subsequently shown to be polypeptides, and the enzymic release and general properties of these hormones intensively studied. The subject is reviewed in APPENDIX.

a) Urinary kallikrein

Highly purified urinary kallikreins have been obtained from the urine of man (Moriya et al., 1963), horse (Prado et al., 1962), hog (Werle and Trautschold, 1963) and rat (Mares-Guia and Diniz, 1967; Porcelli and Croxatto, 1971). The molecular weight of horse urinary kallikrein has been estimated to be 40,500 (Moriya et al., 1963), while Beraldo et al., (1956) report that dog urinary kallikrein has a molecular weight of 40,000 on the basis of ultrafiltration experiments.

Pierce and Webster (1961) isolated kallidin and bradykinin from incubates containing human urinary kallikrein and plasma. Kallidin was found to be the primary product of the incubate and evidence has accumulated to suggest that urinary and other glandular kallikreins liberate

kallidin (lys-bradykinin) whereas plasma kallikrein and trypsin liberate bradykinin.

Urinary kallikreins from different sources hydrolyse synthetic esters such as TAME, BAME, BAEE and BAPA. During purification of these kallikreins, a constant ratio of esterase to kinin-releasing activity is found, suggesting that both properties reside within the same molecule (Webster and Pierce, 1961; Porcelli and Croxatto, 1971). However, when urinary kallikreins from different sources are compared, the ratio of esterase to kinin-releasing activity shows great variation.

b) Urinary kallikrein excretion

The daily output of kallikrein in the urine remains fairly constant in both individual humans (Beaven et al., 1971) and dogs (Beraldo et al., 1956), however, as reported by Frey et al., (1968) there is a wide variation in kallikrein excretion when individuals are compared. More kallikrein is found in the urine collected during the day rather than at night (Elliot and Nazum, 1934), and also in samples collected in the summer rather than winter (Kraut et al., 1934; Werle and Korsten, 1938).

The term "excretion" as applied to urinary kallikrein has been widely used in the literature, however, as the functional role of urinary kallikrein has not been established, it remains to be demonstrated whether or not the substance is an excretion, or in fact an active secretory product. The role of kallikreins in saliva and pancreatic juice

is also unknown, however these enzymes are "true" secretions. Insufficient evidence is available to show that urinary kallikrein is an active secretory product of the kidney, although this would seem likely.

Beraldo et al., (1956) showed that urine formed by a dog heart-lung-kidney preparation perfused with blood, continued to contain kallikrein, although in a reduced concentration. They concluded that urinary kallikrein must either be synthesized in the kidney or derived from plasma kallikrein.

Semb and Nustad (Nustad, 1970 a) investigated the urine formed by an isolated rat kidney perfused with an artificial medium (modified Eagle basal medium). They found kallikrein only in the initial sample collected, subsequent samples were devoid of the enzyme but contained high levels of kininase. Very low levels of urinary kallikrein associated with elevated kininase activity has also been found in patients with normal kidneys undergoing open-heart surgery (Ofstad and Nustad, 1969). Werle et al., (1968) found that urinary kallikrein output increases when a renal transplant is well tolerated, but decreases at the beginning of rejection.

Werle and Vogel (1960) demonstrated that the urine of rats, in which kidney damage had been induced with uranylacetate or salyrgan, contained inactive prekallikrein, while active kallikrein disappeared. If the damage was transient, active kallikrein reappeared upon recovery.

Prekallikrein was also found in the urine of patients with nephrosis. They concluded that damage to the renal tubules by these agents either prevented the formation of urinary kallikrein by the kidney, or prevented its transformation from a plasma kallikrein precursor. It is assumed that the prekallikrein found in the urine during these studies was derived from the plasma.

A relationship between urinary kallikrein excretion and hypertension was demonstrated by Elliot and Nazum (1937) and Frey et al., (1950), finding diminished concentrations in hypertensive compared to normal patients. Croxatto and San Martin (1970 a and b) found that simple removal of one kidney led to a 65% reduction in urinary kallikrein of Wistar rats and 45% in Sprague-Dawley rats. Similar results were also found when one kidney was rendered ischaemic by a figure-of eight - ligature, in this case the contralateral kidney was not disturbed and the animals remained normotensive. However, when the contralateral kidney was removed, the presence of the ligature led to a variable degree of hypertension. In those cases where the hypertension was mild, kallikrein excretion was only slightly less than that of the uninephrectomised normotensive rats. However, severe hypertension (155 mms of mercury or more) led to a reduction in excretion to almost undetectable levels. In some cases, the hypertension fluctuated from week to week, and similar variations in kallikrein excretion were observed. It was concluded that there is an inverse relationship

between blood pressure and the urinary excretion of kallikrein.

Margolius et al., (1972) found that after unilateral renal artery constriction in the rat, hypertension developed even in the presence of the contralateral normal kidney. Kallikrein was undetectable in the urine of six out of seven rats after a period of seven weeks. Although excreting less kallikrein, these animals voided similar volumes of urine over a 24 hour period to sham operated controls, while the total urinary protein was in fact increased.

Margolius et al., (1972) studied a strain of spontaneously hypertensive rats (NIH-F_{21-23} generation Wistar), and found that they excreted between two and three times more kallikrein than normal animals. It was also found that in rats, with hypertension induced by desoxycorticosterone acetate and a high salt intake, five to six times more kallikrein was found in the urine, as compared to control animals.

Collectively, these findings require confirmation and more extensive study, however, it is evident that the relationship between urinary kallikrein and hypertension is complex. The reduced excretion of kallikrein during unilateral renal ischaemia may be a reflection of generalised renal damage, whereas this is not likely in the case of spontaneously hypertensive and DOCA-salt induced hypertensive animals. This explanation is supported by the experiments with isolated and also transplanted kidneys where

reduced excretion was noted, suggesting the susceptibility of the enzyme system to renal damage.

c) Renal kininogenase

The isolation and study of renal kininogenase is a relatively recent development, and the properties of this enzyme(s) have not been characterized as thoroughly as those of the other kallikreins. Although evidence to be presented suggests that this enzyme is typical of a kallikrein, the name "kininogenase" has been adopted until the properties of the enzyme have been further investigated.

A small amount of kininogenase was demonstrated in renal extracts by Kraut et al., (1930) and Werle and Vogel (1961). The properties of the enzyme were first described by Carvalho and Diniz (1964, 1966) following its extraction in an inactive form from rat kidneys. Treatment with acetone, hypotonic sucrose, freezing and thawing, and vigorous homogenisation were all found capable of producing activation. Activated extracts were then incubated with Horton's kininogen substrate in the presence of a kininase inhibitor (8-hydroxyquinoline), and measurable kinin release obtained.

The kininogenase prepared by Carvalho and Diniz did not dialyse through cellophane, was precipitated by 80% v/v acetone or 50% saturated ammonium sulphate, and was destroyed by heating at 90°C for 10 minutes at pH 7.0. The pH optimum for kinin release was 10.0, whereas esterase activity was optimal at pH 6.5 Carvalho (1970) suggests that the difference in pH optima may be due to the very

different nature of the substrates involved.

The activity liberated by the incubates was typical of kinin hormones, causing contraction of the guinea-pig ileum and rat uterus, relaxation of the rat duodenum, and a fall in arterial blood pressure of both cats and dogs. The peptide was indistinguishable from bradykinin using bio-assay and filter paper electrophoresis. It behaved like bradykinin when submitted to the action of proteolytic enzymes, being destroyed by chymotrypsin and B.jararaca venom, but unaffected by trypsin. On the basis of these indirect experiments, the active material released was either bradykinin or a closely related kinin such as kallidin.

Nustad (1969, 1970 a) prepared rat renal kininogenase by gel filtration with Sephadex G-100. This technique separated the kininogenase from kininase activity and provided a semi-purified preparation. Kininogenase activity was determined by incubation with a substrate similar to that of Jacobsen's substrate 2 (Jacobsen, 1966). Kinin liberation was measured using the isolated rat uterus.

d) <u>The relationship between renal and urinary kininogenase</u>

Carvalho and Diniz compared the properties of rat urinary and renal kininogenase, finding that they both behaved similarly in the presence of trypsin inhibitors, but differed significantly from plasma kallikrein. They were inhibited by parotid kallikrein inhibitor (Trasylol) but only partially affected by soya bean trypsin inhibitor

in concentrations that were more effective against plasma kallikrein.

Carvalho (1970) found that Sephadex G-200 gel filtration failed to distinguish between the two enzymes suggesting that they have similar molecular dimensions. Nustad (1969, 1970 a) found similar elution patterns, estimating the molecular weights of both enzymes to be 38,500. Nustad also found both enzymes similar in all properties investigated, liberating kinins from dog and rat plasma, being inhibited by Trasylol and only partially affected by soya bean trypsin inhibitor and ovomucoid trypsin inhibitor. Both enzymes demonstrated a pH optimum of 8.5 for both kinin releasing activity and esterase activity.

e) The macroscopic localization of rat renal kininogenase

Nustad (1969, 1970 b) found kininogenase activity primarily within the renal cortex, although detectable levels were present in the medulla. The cortex-medulla ratio was 8:1 approximately. Most of the activity in the medulla was associated with the outer layer adjacent to the cortex. Kininase activity was equally distributed between the medulla and the cortex.

f) The subcellular localization of rat renal kininogenase

Carvalho and Diniz (1966) using differential centrifugation, fractionated renal homogenates by the procedure of DeDuve et al., (1955). Using acid phosphatase

as a lysosome marker they found kininogenase associated with a "light mitochondrial fraction" which they believed to contain lysosomes.

Nustad (1969, 1970 b) reported different results. He found that kininogenase was associated with a "microsomal" fraction rich in endoplasmic reticulum. Nustad offers an explanation for these contradictory findings on the basis of studies by Erdos and Yang (1966), who found kininase activity associated with the microsomal fraction. The presence of kininase may have prevented the demonstration of kininogenase in this component by Carvalho and Diniz. It is also likely that the "light mitochondrial fraction" studied by Carvalho and Diniz contained a microsomal component as a contaminant.

Subfractionation of the microsomal component led Nustad to conclude that kininogenase, glucose-6-phosphatase and alkaline phosphatase, although being associated with endoplasmic reticulum, were not distributed homogeneously throughout the membrane components of renal homogenates. Evidence from histochemical studies primarily localises glucose-6-phosphatase and alkaline phosphatase to the proximal tubule of the nephron. These findings, together with the observation that the cortex consists of 85% proximal tubular tissue, was cited as evidence to support the concept of a tubular localization of renal kininogenase.

4. Summary

Several unrelated hypotensive substances, including kinin-forming enzymes have been isolated from the mammalian kidney. The role of these substances in the antihypertensive and other functions of the normal kidney remains to be clarified. The properties of renal kininogenase have been described and similarities to urinary kallikrein emphasized. Kininogenase is primarily found in the renal cortex of the rat, and evidence has been presented that suggests a localization within the proximal tubules.

It cannot be stated with certainty that urinary kallikrein is derived from renal kininogenase, although a variety of procedures have demonstrated that following renal damage a diminished urinary excretion is observed. The active secretion of urinary kallikrein by the kidney, if established, would be analagous to the situation found in the pancreas and salivary glands.

The variations in urinary kallikrein excretion produced by various forms of hypertension remain to be explained. Where renal damage was induced by figure-of-eight ligatures or renal artery constriction, depressed urinary kallikrein excretion occurred, whereas in hypertensive states without primary renal involvement, increased excretion was noted.

5. Objectives of the Present Study

a) The development of a reliable technique for the

investigation of kinin-forming enzymes in crude renal extractions.

b) The determination of tissue concentrations and properties of kininogenase in the kidneys of several mammalian species.

c) To localize this activity to a particular component tissue of the kidney.

d) To evaluate the renal kininogenase concentration under experimental conditions known to alter the activity of the renin-angiotensin system.

METHODS

1. Animals and Anaesthesia

a) Dogs (8 - 12 kg.)

Dogs were fed a commercially available dog food and tap water. Anaesthesia was induced by an intravenous injection of sodium pentobarbital (Nembutal), (30 - 35 mg./kg.). Further doses of 30 mg. were administered as required through a femoral vein cannula. A tracheal cannula was inserted to maintain the airway and to provide a rapid route for artificial respiration if the need arose.

b) Rats (200 - 400 gm.), Wistar

Rats were supplied with "Rockland" feed and tap water. Anaesthesia was induced by an intraperitoneal injection of sodium pentobarbital (30 mg./kg.), this dose usually being sufficient to maintain surgical anaesthesia. When duodenal tissue was required for bio-assay purposes, a segment approximately two inches long was removed, washed with oxygenated De Jalons solution and utilized immediately. In all other cases, rats were prepared for renal kininogenase experiments as described below.

c) Guinea-pigs

Random bred guinea-pigs were killed by a blow on the head. A section of terminal ileum approximately 8 inches long was carefully removed, washed and stored in oxygenated magnesium-free Tyrodes solution at 4°C. As required, sections of ileum one inch long were utilized for the assay procedure.

2. The Preparation of Kidney Tissue

a) Preliminary surgery (dogs and rats)

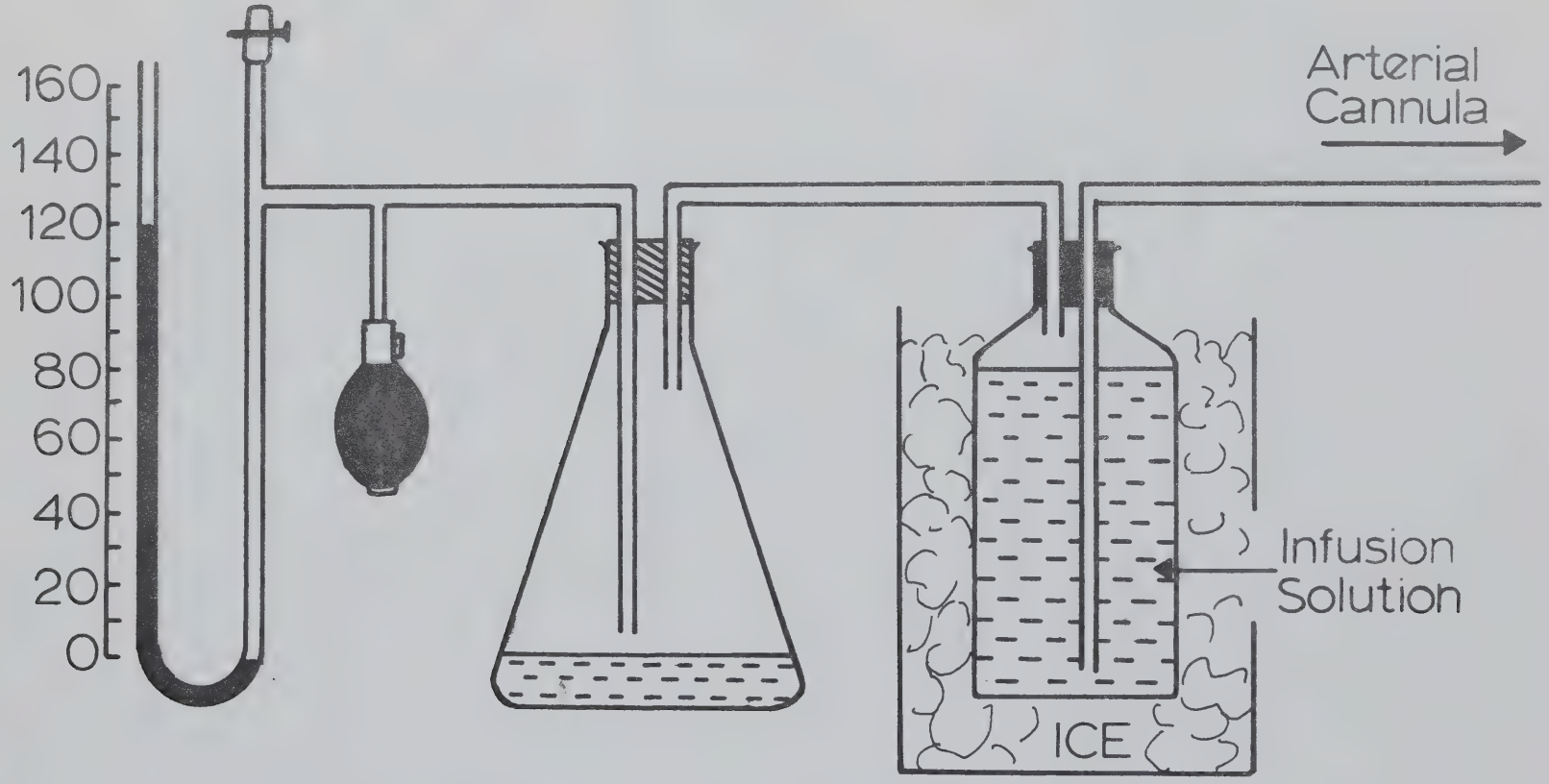
A mid-line incision was made through the abdominal wall extending from just below the xiphoid process of the sternum to the pubic symphisis. The abdominal wall was retracted and the abdominal contents manipulated to allow access to the renal arteries and veins, which were then exposed by blunt dissection.

The aorta was exposed at the femoral bifurcation, and also at the origin of the two renal arteries. The mesenteric arteries were tied off, and the vena cava exposed. During a brief period of aortic occlusion below the kidneys to prevent renal ischaemia, the aorta was cannulated near to the femoral bifurcation.

The animal was then heparinised (Heparin Sodium, Nutritional Biochemical Corporation, 10 mg./ml. dissolved in isotonic saline, in a dose of 5 mg./kg.) and the aorta tied off above the origin of the renal arteries. The vena cava was cut and the kidneys perfused via the aortic cannula with 0.25M sucrose solution (De Carvalho and Diniz, 1964).

Perfusion was considered satisfactory when the venous effluent became clear and the kidneys assumed a pale straw colour. Perfusion pressure was maintained between 120 to 100 mms. of mercury with the apparatus shown in Figure 1. During dog experiments, it was often found advantageous to perfuse the kidneys "in vitro" by excising them together with an inch of renal artery, the artery was

FIGURE 1: The infusion pump



INFUSION PUMP

then cannulated and the kidneys perfused in a beaker surrounded by ice water. This was a matter of convenience and appeared to have no influence on the success of the procedure.

b) Rat cortex-medulla separation

After perfusion with sucrose, the kidneys were cut into sections approximately 2 mms. thick. The demarcation between cortical and medullary tissue was always clear and facilitated careful separation. The cortical and medullary tissues from each kidney were placed in labelled glass vials and lyophilised (Virtis Refrigerated Lyophiliser). The dried samples were then stored at -20°C.

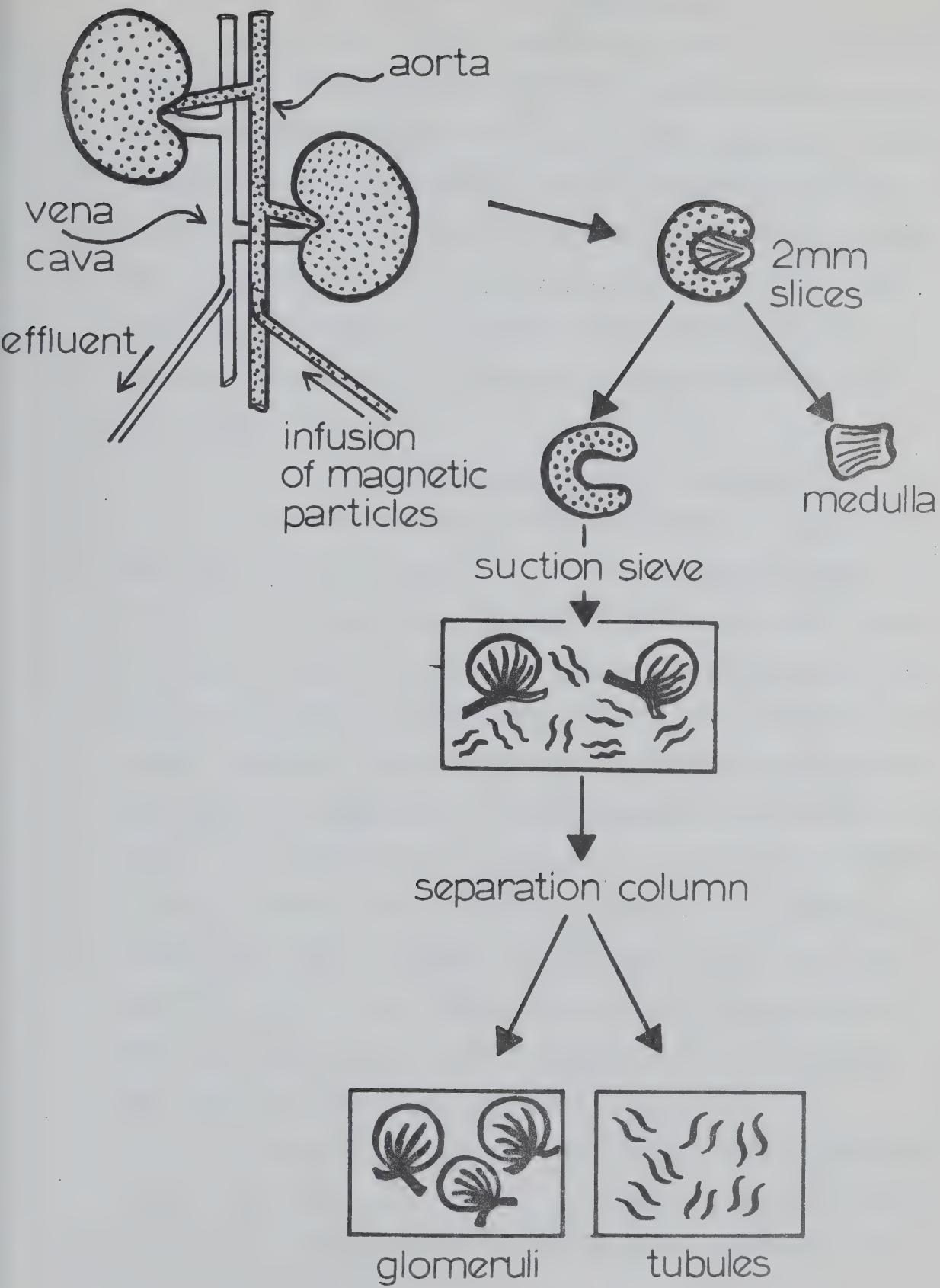
c) The isolation of glomeruli from dog kidney

The isolation of glomeruli in sufficient quantities for bio-assay was first described utilizing the rabbit kidney by Cook and Pickering in 1959. A modification of this technique applicable to the dog was devised, and is summarized in Figure 2.

i. Preparation of iron particles

2.6 gms. of sodium hydroxide and 20 gms. of potassium nitrate were dissolved in 100 mls. of distilled water. The solution was then poured into a solution of 9 gms. ferrous sulphate in 100 mls. of distilled water. The mixture was brought to its boiling point, the precipitate obtained being separated and washed ten times with distilled water by decantation. The fine particles of iron oxide were stored in isotonic saline until required.

FIGURE 2: The isolation of glomeruli of the dog kidney


aorta
vena cava
effluent
infusion of magnetic particles
2mm slices
medulla
suction sieve
separation column
glomeruli
tubules

ii. Perfusion of magnetic particles

After the kidneys had been perfused with cold sucrose solution, a suspension of iron oxide particles was perfused. The suspension contained 40 mgs. of iron oxide per 100 mls. of isotonic saline, unlike Cook and Pickering who used a 7% gum acacia suspension. Perfusion was considered complete when the cortex became uniformly infiltrated with black deposits. Although no accurate record of the volume of perfusate was taken, it was approximately 500 mls. for each kidney.

iii. Separation of renal fractions

Cook and Pickering (1959) presented evidence to show that the iron oxide particles are sufficiently minute to penetrate the glomerular capillary tuft where they become entrapped. They are unable to penetrate further into the capillary networks around the cortical and medullary portions of the nephrons. When the cortical tissue is fragmented through a 160 mesh sieve, the glomeruli, many of which remain intact, are physically dissociated from the other cortical tissues. These iron containing glomeruli are magnetic, whereas the other elements of the renal cortex are non-magnetic. It is the magnetic property of these glomeruli that forms the basis of their separation in sufficient quantities for bio-assay purposes.

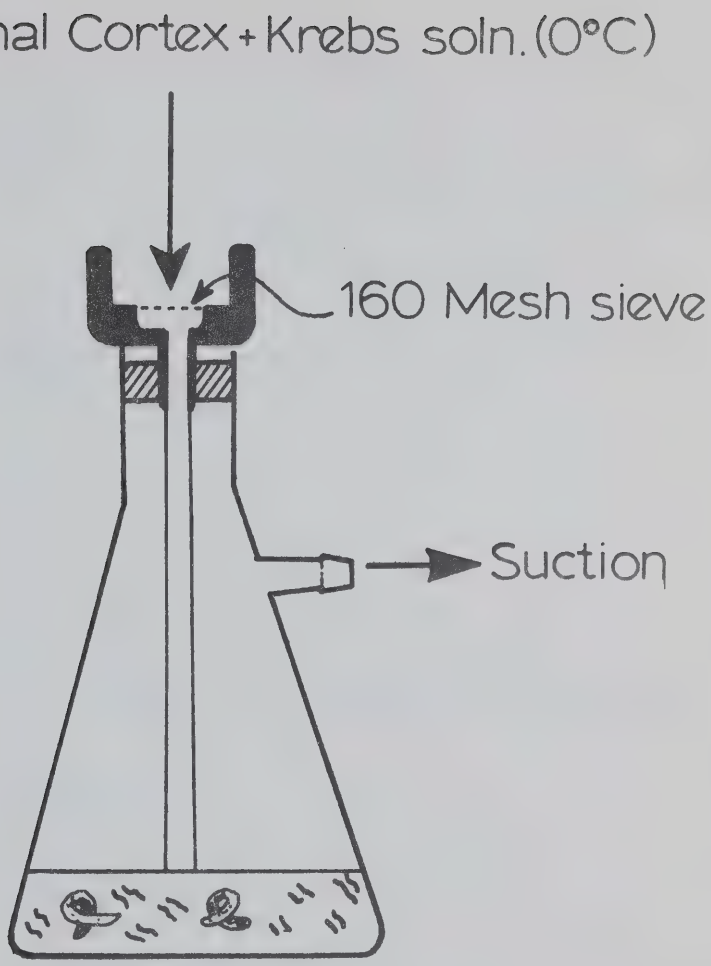
A perfused kidney was kept for no longer than an hour at 4°C before the capsule was removed and the kidney sliced with a scalpel into sections approximately 2 mms.

thick. At this point, a sample of renal tissue was taken for histology to check the effectiveness of the perfusion. The cortex was then carefully dissected from the medulla of each slice, the medullary tissue being collected, frozen in dry ice and acetone, and lyophilised.

The cortical tissue was then cut into smaller portions which were individually placed in a Sietz 20 ml. filter (Fisher Scientific Company) containing a 160 mesh stainless steel sieve (W. Tyler Company). The fragments were forced through the apertures of the sieve with the flat end of a plunger from a 20 ml. glass hypodermic syringe, the process being facilitated by vacuum as shown in Figure 3. The tissue was washed through the sieve with cold Krebs solution. A small amount of fibrous tissue was always retained in the filter and was discarded. The entire renal cortex was processed in this manner accumulating as a very coarse suspension in the collecting flask.

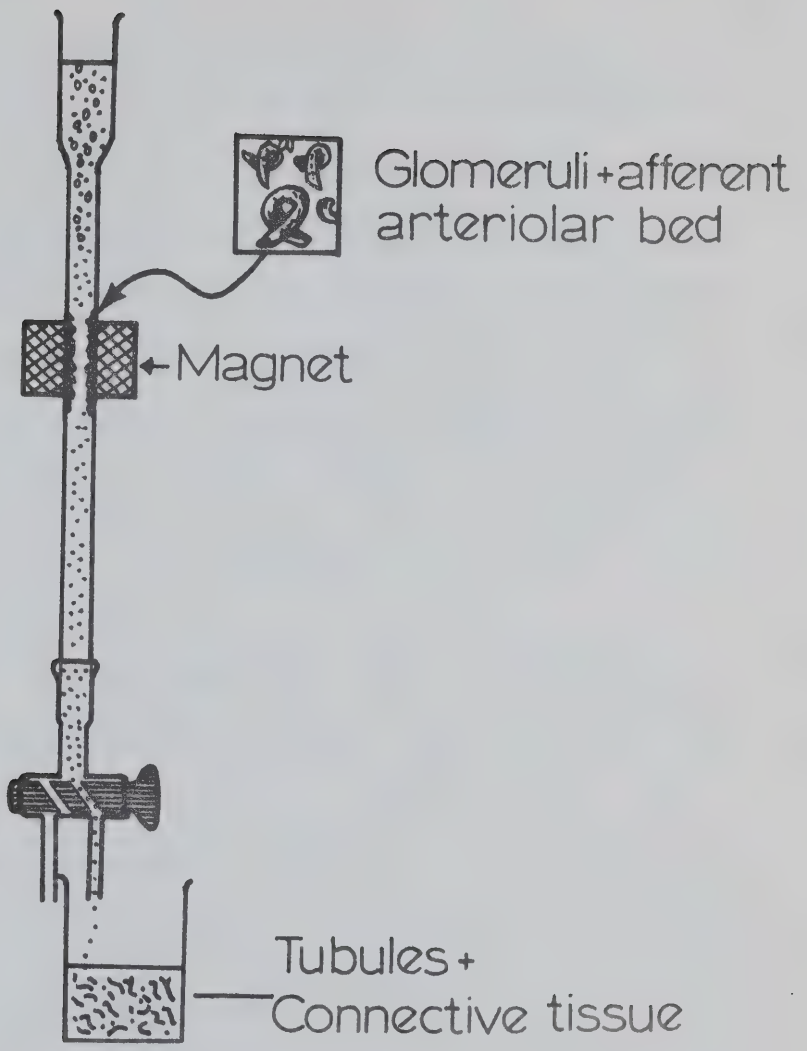
The suspension of cortical tissue was then applied to a glass column as illustrated in Figure 4. The column was modified from a 50 ml. burette, with a two way tap to regulate the rate of flow of the sample. Midway along the column, a large magnet was fixed with its poles surrounding the wall of the cylinder. 50 ml. aliquots of the suspension were applied and given 5 minutes for the magnetic components to aggregate and adhere to the column wall adjacent to the magnet. On opening the tap, the suspension was allowed to drain slowly into a beaker at a rate which did not dislodge

FIGURE 3: Fragmentation of the renal cortex



FRAGMENTATION OF THE RENAL CORTEX

FIGURE 4: Separation column for the isolation of magnetic particles



SEPARATION COLUMN FOR ISOLATION OF MAGNETIC PARTICLES.

the adherent particles. In this way, the tubular and connective tissue elements of the cortex passed into the beaker leaving the magnetic glomerular tissues attached to the column wall. The glomerular fraction was then washed into a separate beaker with approximately 20 ml. of Krebs solution. This procedure was repeated until the entire cortical suspension had been separated. In this manner, two fractions were collected, hereafter called the "glomerular" and "tubular" fractions. However, it should be understood that this terminology is used to imply that glomeruli and tubules respectively, are the major but not sole components of these two fractions.

The first separation was always incomplete, considerable cross-contamination being present. The separate fractions were thus individually re-applied to the column three or four times. A considerable quantity of contaminants were always recovered in this way. The purity of the separation was checked by pipetting a small sample of each fraction onto a microscopic slide and observing the tissues under low power light microscopy. The effectiveness of the separation judged in this manner was always subjective, and no strict criteria were laid down. It was deemed complete when isolated glomeruli appeared to be absent from the tubular specimen and the glomerular fraction appeared homogeneous.

When the separation was completed, both fractions occupied large volumes of Krebs solution, often up to

500 mls. After centrifugation for 30 mins. at 10,000 r.p.m. and 4°C (Lourdes refrigerated centrifuge) the large volume of supernatant was discarded. A small sample of each pellet was taken for histology and the remaining tissue lyophilised and stored at -20°C.

3. Histological Studies

A sample of intact renal tissue was taken to demonstrate the degree of penetration of the iron particles into the glomerular tissues. Also, samples of the final glomerular and tubular fractions were taken prior to lyophilisation to assess their purity.

a) Intact, iron perfused renal tissue

i. Fixation - The specimens were fixed overnight in buffered neutral formaline at 4°C.

Composition - 40% formaldehyde	100	mls.
- distilled water	900	mls.
- monobasic sodium phosphate	4	gms.
- anhd. dibasic sodium phosphate	6.5	gms.

ii. Wash - Overnight in running tap water.

iii. Dehydration - 1/2 to 2 hours in each of the following sequence of alcohol solutions:

50, 70, 80, 95, 98 and 98% ethanol

iv. Clearing - The specimens were taken through two changes in benzene, each for one half hour

duration.

v. Embedding - After two changes, each for one half hour in melted Tissuemat M.Pt 56-58°C (Fisher Scientific Company), the samples were finally embedded in celluloid moulds.

vi. Sectioning - Sections were cut at 5 to 10 microns thick with a Leitz microtome. The sections were floated as ribbons on a Lipshaw thermostatically controlled water bath at 45°C, and picked up on a slide smeared with Meyer's albumin solution.

vii. Drying - The slides were left to dry overnight in an oven at 45°C.

viii. Staining - Delafield's (or Harris) Haematoxylin by the progressive method, as described by Humason (1967).

xylene	2-3 mins.
xylene	2-3 mins.
98% ethanol	2-3 mins.
95% ethanol	2-3 mins.
70% ethanol	2-3 mins.
running tap water	5 mins.
Haematoxylin	2-5 mins., with check for depth of stain
running tap water	5 mins.
Scott's solution	3 mins.
running tap water	5 mins.
Eosin	1 or more minutes with check for the depth of stain.

ix. Mounting - The specimens were dehydrated and prepared for the mounting medium (Permount, Fisher Scientific Company) as follows:

70% ethanol	1 or more dips
95% ethanol	2 dips
98% ethanol	3 mins.
Xylene	3 mins.
Xylene	3 mins.

b) Glomerular and tubular fractions

i. Fixation - A sample of each pellet was fixed overnight in Bouin's fixative:

composition -	1% picric acid	75 mls.
-	formaline	25 mls.
-	gl. acetic acid	5 mls.

ii. Dehydration and embedding - The fixed material was spun-down in a centrifuge tube. The fixative was removed by decantation and 50% ethanol added to the pellet and left for 15 minutes with occasional agitation. The alcohol was removed by centrifugation and the procedure repeated with the following sequence of solutions, each for 20 minutes:

50, 70, 80, 95, 98 and 98% ethanol

benzene

The dehydrated and cleared cellular suspensions were again spun down in a centrifuge and the benzene decanted off. Melted tissuemat was then added and the pellet of wax containing the particulate matter was allowed to solidify

in the centrifuge tube. The tube was then carefully shattered, and the paraffin block sectioned, stained and mounted as described previously.

c) Microphotography

35 mm. colour slide photographs were taken on Kodachrome film using a Zeiss Photomicroscope II.

4. The Determination of Renal Kininogenase Concentrations

Renal kininogenase concentrations were determined by the quantitative measurement of kinin release following the incubation of renal extracts with a suitable substrate. Isolated guinea-pig ileum was used for the bio-assay procedure.

a) Preparation of the substrate, dog pseudoglobulin

The following procedures were carried out utilizing polyethylene beakers and containers, at no time was the plasma allowed to come into contact with glass surfaces.

Freshly collected heparinised dog plasma, or a 7% solution of lyophilised dog plasma (Nutritional Biochemicals Corporation) in isotonic saline was heated for two hours at 56 to 58°C in a water bath. The precipitate was removed by centrifugation for 20 minutes at 10,000 r.p.m., 4°C, and the supernatant dialysed against isotonic saline overnight at 4°C. An equal volume of saturated ammonium sulphate was added to the dialysed plasma drop by drop with constant stirring at 4°C. After approximately two hours, the precipitate was collected by centrifugation for 20 minutes

at 10,000 r.p.m. The precipitate was then dissolved in distilled water, the volume required being either one third the plasma volume in the case of freshly collected plasma, or 4 mls. of distilled water for every gm. of lyophilised dog plasma. The solution was then dialysed for 24 hours at 4°C against tap water, followed by 24 hours against distilled water. The precipitate was collected, lyophilised and stored in polyethylene containers at -20°C until required.

b) The extraction of renal kininogenase

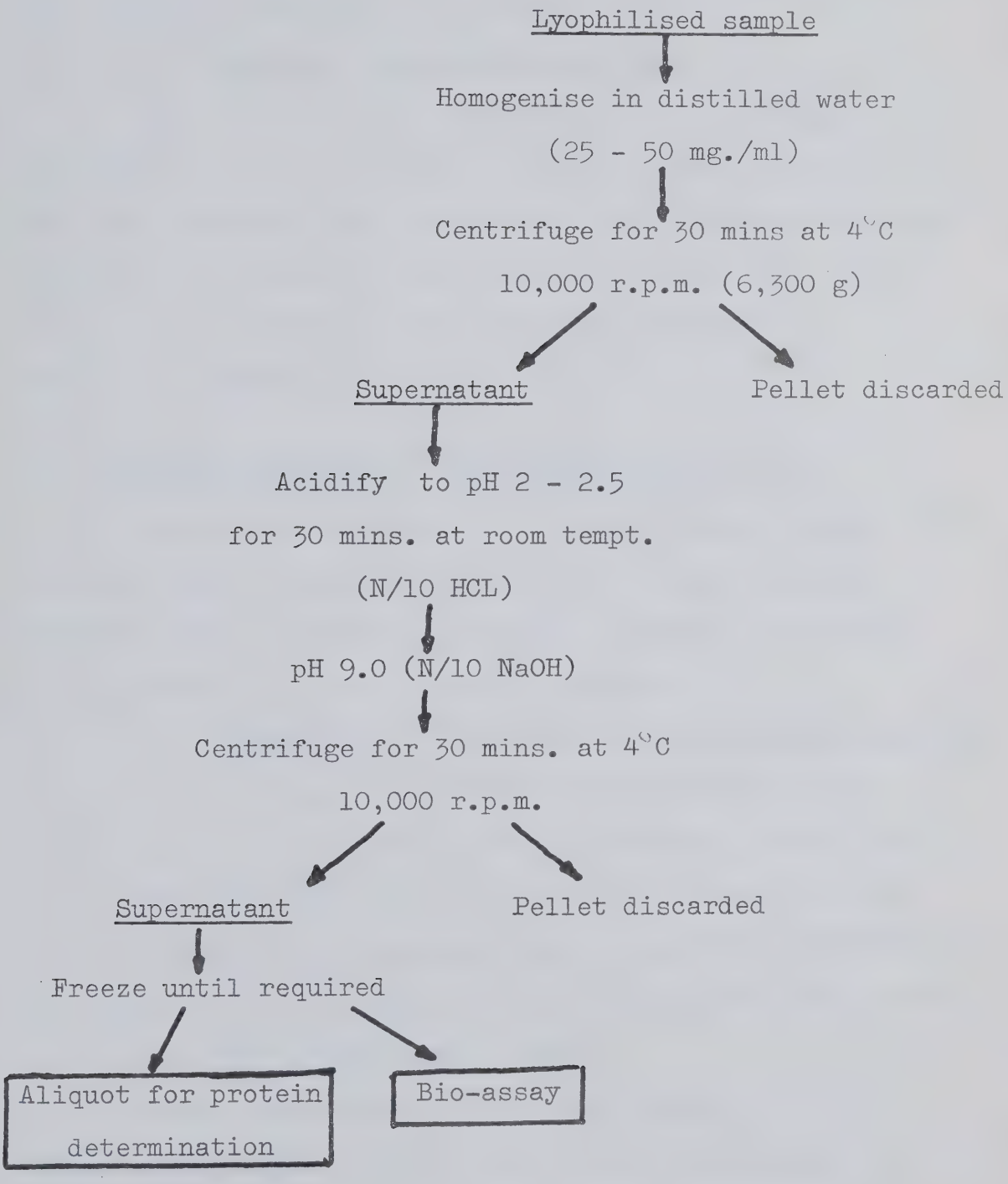
A series of preliminary experiments were carried out as described under RESULTS. It was found that acidification of renal homogenates to pH 2.0 - 2.5 for 30 minutes at room temperature inactivated kininase enzymes. On the basis of these studies, extracts were prepared as shown in Figure 5. The extracts were adjusted to pH 9.0 and brought to 37°C prior to incubation.

Optimal conditions for the incubation and liberation of kinins were evolved during the preliminary experiments and are only mentioned briefly below.

c) Incubation conditions

The lyophilised dog pseudoglobulin substrate was dissolved in bicarbonate buffer at pH 9.0 in a concentration of 50 mg./ml. 0.3 mls. were transferred to a 5 ml. glass test tube and placed in a water bath for 5 minutes at 37°C. An aliquot of the kininogenase extract was added, and the solution incubated with occasional agitation for 20 minutes at 37°C.

Figure 5. The extraction of Kininogenase From Renal Tissue



d) Bio-assay of kinin concentrations

Kinin liberated into the incubate was determined by the procedure described in section 6 below.

e) Units of kininogenase activity

The activity of a renal extract was expressed as the amount of kinin liberated per milligram of protein in the renal extract after incubation with dog pseudoglobulin under the experimental conditions described. The kinin liberated was expressed as micrograms of synthetic bradykinin (Sandoz BRS-640).

5. The Determination of Renal Renin Concentrations

Renin was determined by the quantitative measurement of angiotensin release following the incubation of renal extracts with a suitable substrate. Isolated guinea-pig ileum was used for the bio-assay procedure.

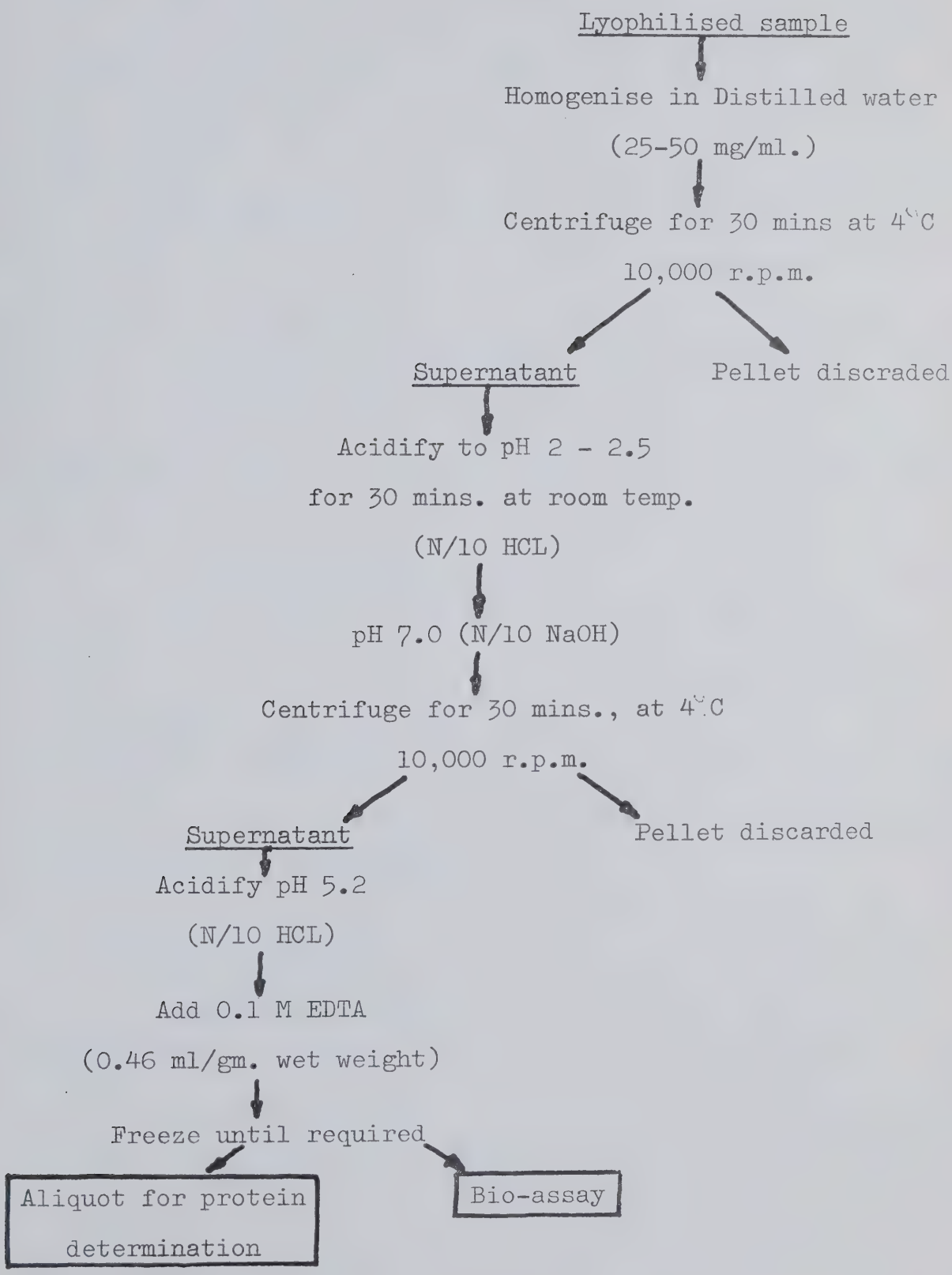
a) Preparation of the substrate, dog renin substrate

A dog was nephrectomised and maintained under sodium pentobarbital anaesthesia for 8 hours. After this time, the animal was heparinised and the blood collected into a polyethylene container from a femoral artery cannula. The whole blood was centrifuged and the plasma lyophilised.

b) The extraction of renin

Renin was extracted by the procedure of Gross, Brunner and Zeigler (1965). The method is summarised in Figure 6. 1 ml. aliquots of the extract were placed in 5 ml test tubes and brought to 37°C in a water bath prior to incubation.

Figure 6. The Extraction of Renin



c) Incubation conditions

The lyophilised substrate was dissolved in distilled water (50 mg./ml.) and 1 ml. transferred to a 5ml. glass test tube and brought to 37°C. To this was added 1 ml. of renal extract and the mixture incubated for one hour at 37°C. The mixture was then boiled for 10 minutes to inactivate the enzymes present, and the supernatant obtained by centrifugation for 30 minutes at 15,000 r.p.m.

d) Bio-assay of angiotensin concentrations

Angiotensin liberated into the incubate was determined by the bio-assay procedure described in section 6, below. Aliquots of the supernatant were utilised.

e) Units of renin activity

The activity of a renal extract was expressed as the amount of angiotensin liberated per milligram of protein when incubated with dog renin substrate under the experimental conditions described. The angiotensin liberated was expressed as micrograms of synthetic val^5 angiotensin II (Hypertensin, Ciba Laboratories Ltd.).

6. The Assay Procedure

A commercially available isolated organ bath (C.F. Palmer Limited, model 6110/04) was used, consisting of a perspex bath 12 x 9 inches and 8 inches deep. The bath was mounted on a sturdy angle iron frame with adjustable clamps on the top for holding a force-displacement transducer. Around the lower inside edge of the bath was a 250 watt

thermostatically controlled heating element. When the bath was filled with tap water, the temperature was regulated between 30-32°C.

The smooth muscle was suspended in an 8 ml. chamber, and bathed in either Tyrode's or De Jalon's solution depending upon the tissue under investigation. The solutions contained atropine and mepyramine (both 2 x 10^{-8} gm./ml.) and were gravity fed from a two litre reservoir into two glass equilibrating coils and then into the chamber, in this way the solutions were always at bath temperature before coming into contact with the tissue. The solution was aerated with 95% oxygen/5% carbon dioxide mixture.

The intestinal muscle was tied to the base of the chamber, while the other end of the tissue was tied to a force-displacement transducer (Model FT.032C, 2 mg. to 2 kg. range, Grass Instrument Company). The contractions of the tissue were recorded with a pen recorder on heat sensitive paper (Sanborn Model 7702B 2-channel pen recorder, with an 8805A carrier pre-amplifier, (Hewlett Pakard Company). A load of 4 gms. was applied to the tissue at the beginning of each experiment, and the sensitivity of the pen recorder adjusted to give recordings of a suitable size. The deflections produced were calibrated in gms.

a) Quantitative determination of peptide concentrations

The amount of kinin or angiotensin liberated from incubates prepared as described in sections 4 and 5 above, was determined as follows.

A modification of the four-point assay system described by Rocha E Silva (1970) was utilised and is illustrated in RESULTS. Absolute quantitation of the peptide concentrations is ideally obtained by the statistical analysis of the response of the isolated guinea-pig ileum to repeated dose cycles of renal incubates and standard peptide solutions. Insufficient quantities of the incubates prevented the application of this procedure, however, an absolute quantitation would be difficult with extracts and substrates that are as impure as those used in this study. The objective of the investigation was to compare the relative activities of different renal fractions, which does not require such a refined technique.

The entire incubate, or aliquots of it was injected into the chamber and remained in contact with the guinea-pig ileum for 45 seconds. The response of the ileum was recorded during this period, after which the recorder was stopped and the chamber emptied and washed out with fresh Tyrode's solution. A four minute dose cycle was used, the test solutions (incubates) and standard peptides being administered alternately every four minutes. Wherever possible, the response of the ileum to three different doses of the incubate was compared to three different doses of the standard peptide solution. The doses were adjusted to provide responses that were neither supramaximal or barely detectable. Dose response curves were drawn and from these, the concentration of peptides

in the incubates determined. In most cases this procedure was repeated several times with each extract.

It was found that the guinea-pig ileum performed optimally at a bath temperature of 32°C.

b) Qualitative biological assay of peptides

Rat duodenum was used in studies to identify the type of peptide released by renal extracts. The duodenum was suspended in De Jalons solution containing atropine and mepyramine (both 2×10^{-8} gm./ml.) and aerated with 95% oxygen/5% carbon dioxide gas mixture. A four minute dose cycle was used and the bath temperature set between 30 - 31°C.

7. Protein Determinations

The protein concentration of renal extracts was measured by the method of Lowry et al., 1951. The colour change produced in the presence of protein was quantitatively measured with a Beckman Spectrophotometer. The protein concentration was expressed as the number of micrograms of protein per milligram of lyophilised tissue.

8. Drugs and Other Materials

a) Peptides - Bradykinin was a synthetic preparation kindly supplied by Sandoz Ltd, Basle, Switzerland; Angiotensin II was provided by Ciba Laboratories, Dorval, Quebec.

b) Physiological saline solutions

Tyrode's solution consisted of the following:

Distilled water	5	litres
Sodium chloride	40	gms.
Potassium chloride	1	gm.

Calcium chloride	1	gms.
Sodium bicarbonate	5	gms.
Glucose	5	gms.
Sodium dihydrogen phosphate	0.3	gms.

De Jalon's solution contained the following:

Distilled water	5	litres
Sodium chloride	45	gms.
Potassium chloride	2.1	gms.
Calcium chloride	0.3	gms.
Sodium bicarbonate	2.5	gms.
Glucose	5	gms.

RESULTS

1. Preliminary Investigations

a) The inhibition of kininase

The demonstration of kininogenase in renal tissue relies upon the ability of renal extracts to liberate kinins from a suitable substrate. However, the presence of kininases in renal extracts interferes with this process by destroying kinins before they are detectable by bio-assay procedures.

Several techniques have been described for the inhibition of rat renal kininase. Carvalho and Diniz (1964, 1966) added 0.06M 8-hydroxyquinoline to renal homogenates to reach a final concentration of 2×10^{-3} mMoles/ml. However, although kinin release was demonstrated following this procedure, it was subsequently admitted that the inhibition was incomplete (Carvalho, 1970).

Nustad (1970 a) separated kininogenase and kininase activity into different fractions by Sephadex gel filtration, hence removing this source of interference. In a few experiments with crude renal extracts, Nustad used mercaptoethanol (3×10^{-2}M) and dipyridyl (2×10^{-3}M) as kininase inhibitors, also with inconsistent results.

It was thus necessary to evaluate a variety of procedures likely to inhibit kininase activity. This was carried out by treating renal extracts and then incubating them with known concentrations of synthetic bradykinin. The recovery of the peptide over varying time periods was

then estimated.

i. Procedure

Rat kidneys were perfused with 0.25M sucrose and after mincing were lyophilised. The dry tissue was powdered in a pestel and mortar and homogenised in distilled water (10 mg./ml.). The homogenates were then treated by a variety of procedures as shown in TABLE 1. Aliquots of treated and untreated homogenates were then incubated with several different concentrations of synthetic bradykinin for periods between one and twenty minutes (variable times were used depending upon the effectiveness of inhibition). The recovery of bradykinin was determined by comparing the activity of the incubates against control incubates of standard bradykinin with isotonic saline. The activity of the samples was determined by bio-assay using the guinea-pig ileum.

ii. Results

An example of the results obtained is shown in FIGURE 7 (a and b). This is a tracing of the recordings obtained during an assay series, and illustrates the tabulated findings shown in TABLE I.

Untreated homogenates rapidly destroyed bradykinin, inactivating up to 400 nanograms of the peptide within one to five minutes.

The procedure described by Carvalho and Diniz using 8-hydroxyquinoline was repeated ten times, in each experiment the procedure was found ineffective, bradykinin being almost

Table 1. The inhibition of Kininase

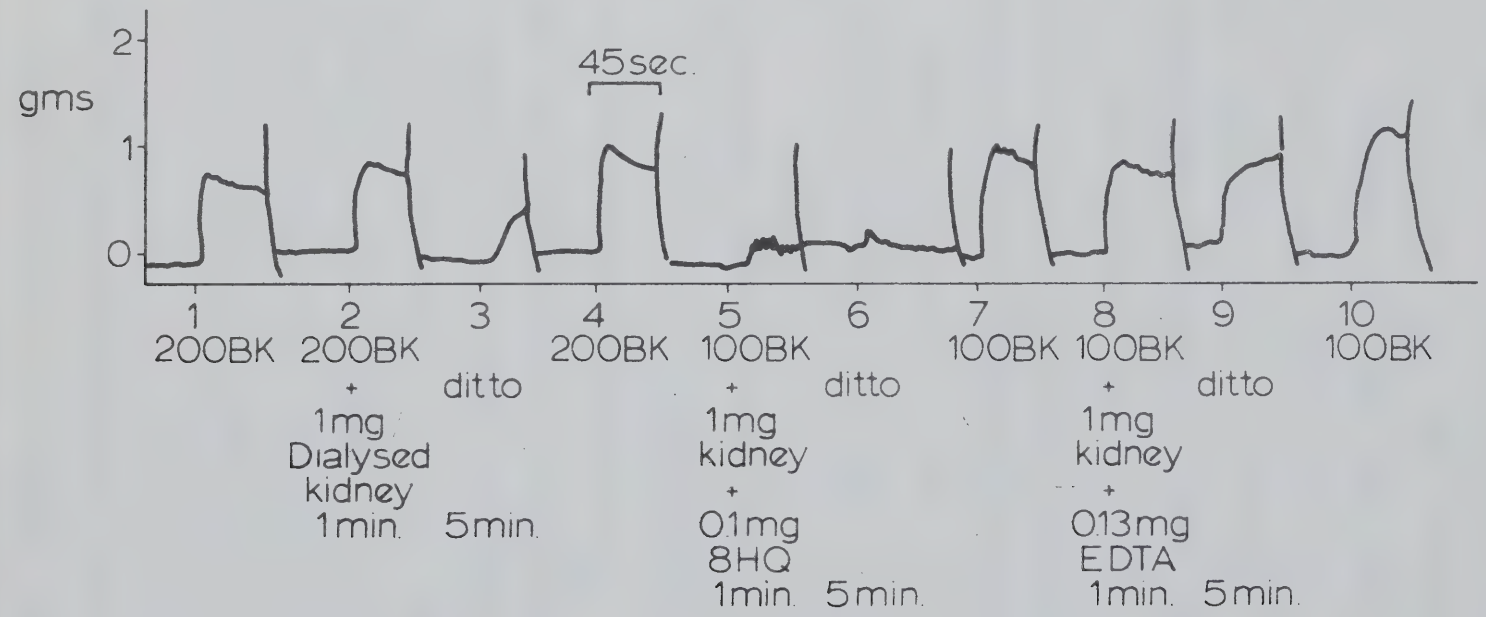
Number of experiments	Bradykinin concentration (nanograms)	Procedure	Incubation Time	% Recovery
7	200 - 400	Untreated	5 min.	0
10	100 - 400	8 hydroxy-quinoline (7×10^{-3} mM/ml in homogenate)	5 min.	0-20
7	100 - 200	Homog. acidified to pH 2.0 for 20 mins. Returned to pH 7.0	20 min.	95-100
4	500	Dialysis of homog. 12 hrs. distilled water	5 min.	0-50
2	100	E.D.T.A. (4×10^{-3} mM/ml in homogenate)	5 min.	90
3	100	Mercaptoethanol (2×10^{-1} mM/ml in homogenate)	9 min.	75
2	100	Sodium bisulphite ($i \times 10^{-4}$ mM/ml in homogenate)	9 min.	0
2	100	Mercuric chloride (4×10^{-5} mM/ml in homogenate)	5 min.	0

FIGURE 7 a

The effect of dialysis, 8-hydroxyquinoline and EDTA on rat renal kininase

Key: BK - bradykinin (nanograms)
ditto - identical incubate composition to previous sample, however, different incubation time.

The tracing shows contraction of the guinea-pig ileum following the administration of test solutions (indicated by event marker). The responses are calibrated in gms. The ileum was suspended in magnesium-free Tyrodes solution containing atropine and mepyramine (2×10^{-8} gm./ml) at 32°C. The vertical deflection of the tracing at the end of each period is a "washing-out" artefact.


gms
2
1
0
45sec.
1
200BK
2
200BK
+
1mg
Dialysed
kidney
1min.
3
ditto
5min.
4
200BK
5
100BK
+
1mg
kidney
+
0.1mg
8HQ
1min.
6
ditto
5min.
7
100BK
8
100BK
+
1mg
kidney
+
0.13mg
EDTA
1min.
9
ditto
5min.
10
100BK

totally inactivated within five minutes.

Of the procedures utilised, acidification of the homogenate (FIGURE 7c) and the addition of ethylenediamine tetraacetic acid (EDTA) provided the most effective inhibition.

On this basis, acidified and EDTA-treated homogenates were then incubated with 15 mg. of dog pseudoglobulin (50 mg./ml. in isotonic saline) at pH 7.0 for between one and twenty minutes. It was found that kinin release was only successfully observed in the case of the acidified homogenates (three experiments), and that kinins were still evident in increasing concentrations after 20 minutes of incubation (FIGURE 7d).

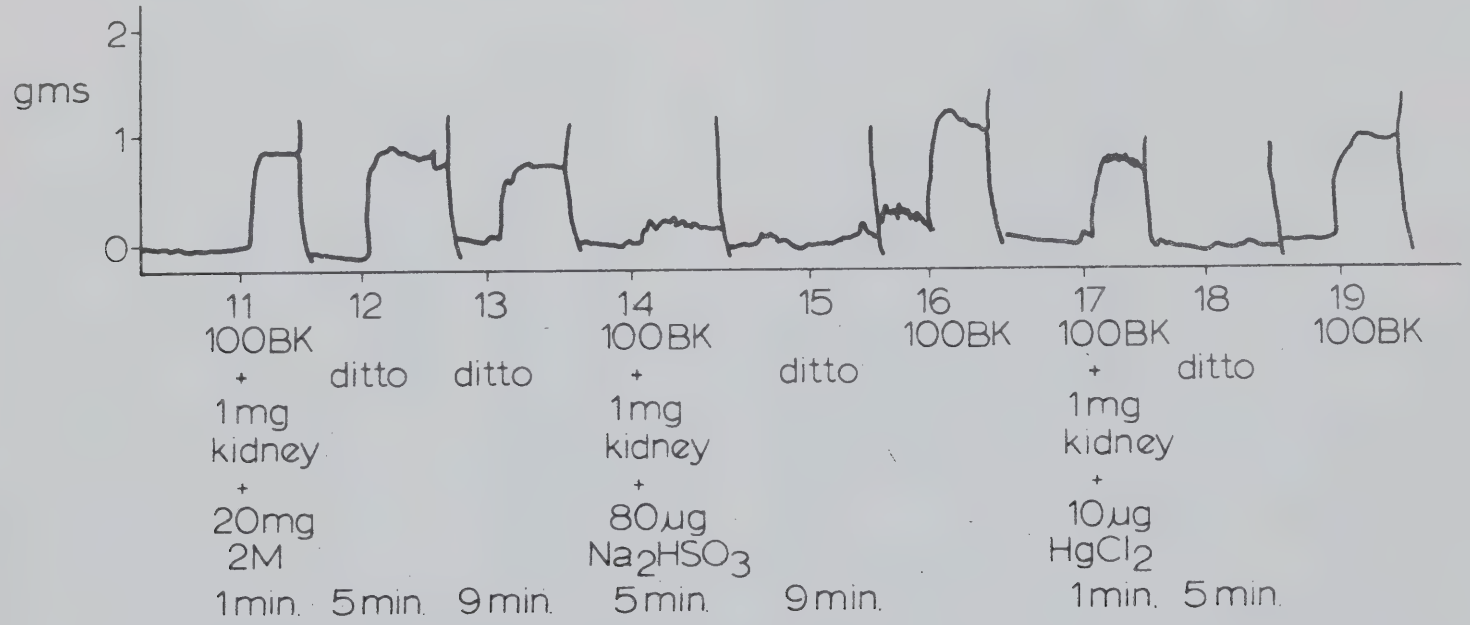
It was thus concluded that acidification of the homogenate to pH 2.0 for 20 minutes, followed by the return of the pH to 7.0 prior to incubation, was the most effective procedure available for the inhibition of renal kininase. The partial effectiveness of 8-hydroxyquinoline and 2 mercaptoethanol reported by other investigators was also found to apply in this study, however it cannot be concluded that these agents are totally ineffective.

Subsequent to these findings, acidification was utilised for the kininase inhibition of renal extracts obtained from rats and other species. It cannot be stated with certainty that total inhibition was present, this being more difficult to evaluate in incubates of unknown kinin-releasing potential. It is suggested that kininase activity

FIGURE 7 b

The Effect of 2-mercaptoethanol, sodium bisulphite and mercuric chloride on rat renal kininase

Key: 2 M - 2 mercaptoethanol

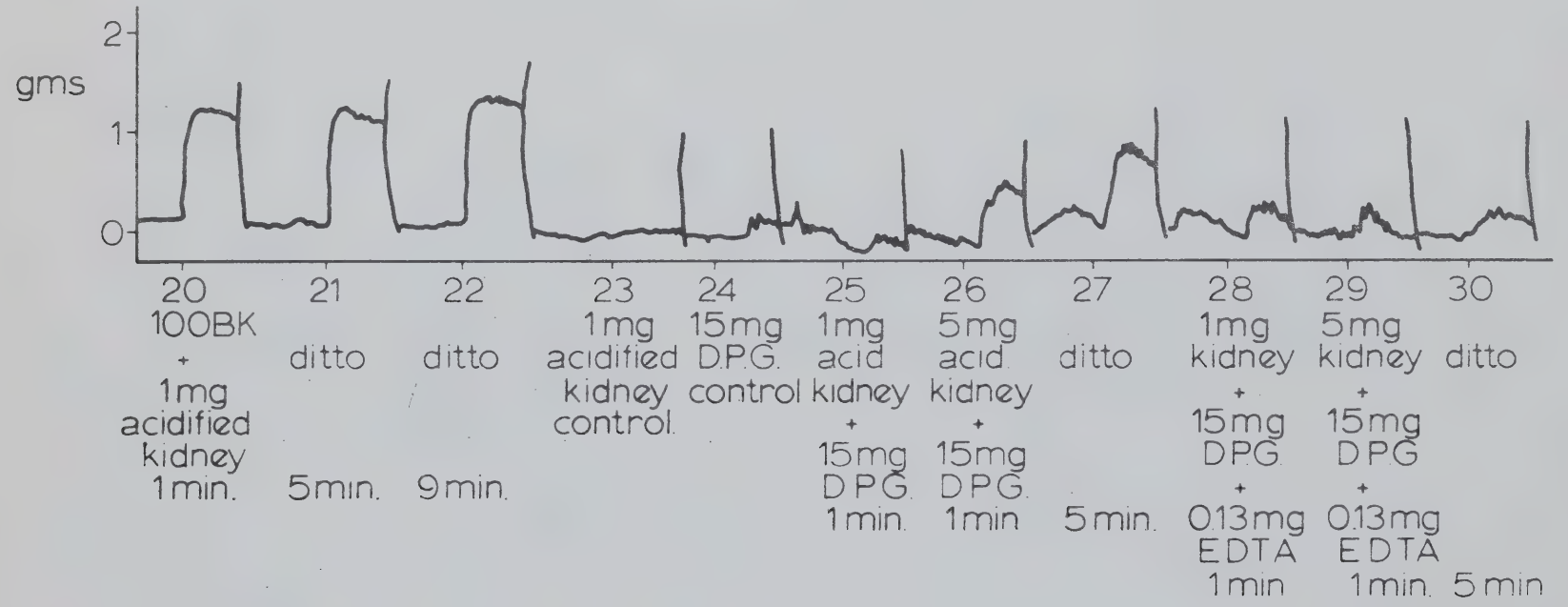

gms
2
1
0
11
100BK
+
1mg
kidney
+
20mg
2M
1min.
12
ditto
5min.
13
ditto
9min.
14
100BK
+
1mg
kidney
+
80µg
Na_2HSO_3
5min.
15
ditto
9min.
16
100BK
17
100BK
+
1mg
kidney
+
10µg
$HgCl_2$
1min.
18
ditto
5min.
19
100BK

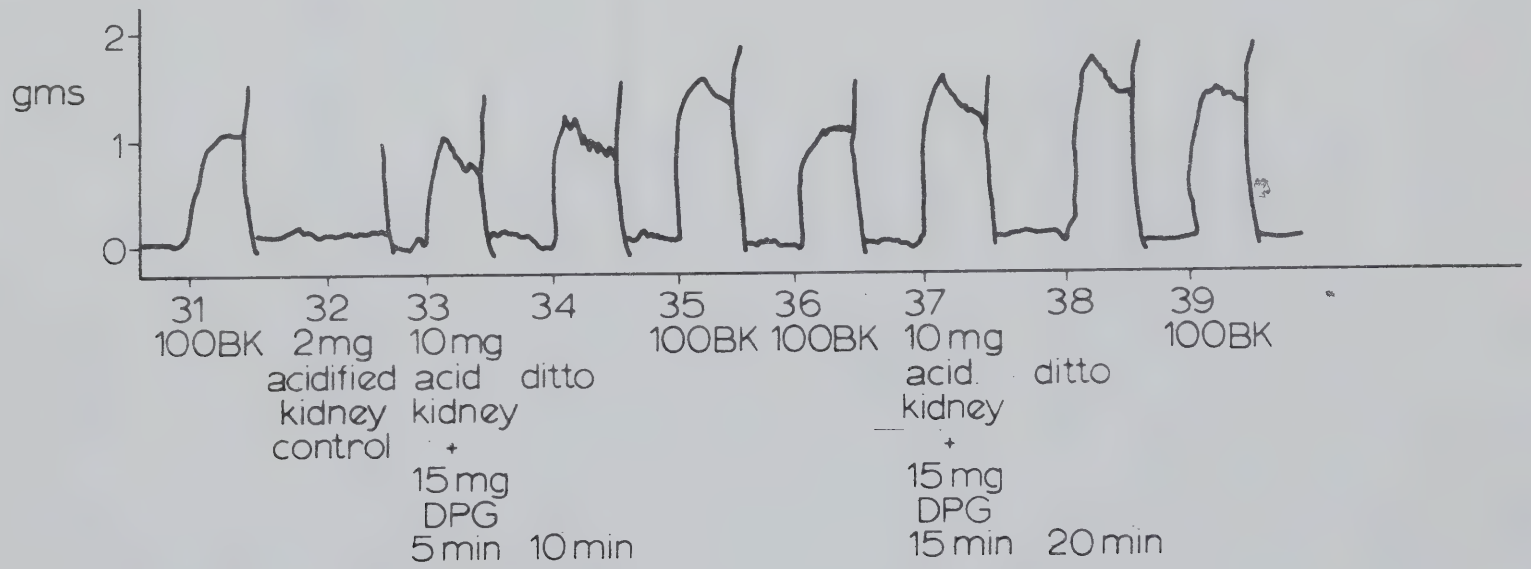
FIGURES 7 c and d

The effectiveness of acidification of renal homoegenates, for the inactivation of kininase

Key: - DPG - dog pseudoglobulin substrate

- acidified kidney control - homogenate incubated with 0.3 mls. of isotonic saline.
- DPG control - substrate incubated with 0.2 mls. of isotonic saline


gms
2
1
0
20
100BK
+
1mg
acidified
kidney
1min.
21
ditto
5min.
22
ditto
9min.
23
1mg
acidified
kidney
control.
24
15mg
D.P.G.
control
25
1mg
acid
kidney
+
15mg
D.P.G.
1min.
26
5mg
acid
kidney
+
15mg
D.P.G.
1min
27
ditto
5min.
28
1mg
kidney
+
15mg
DPG
+
0.13mg
EDTA
1min
29
5mg
kidney
+
15mg
DPG
+
0.13mg
EDTA
1min.
30
ditto
5min


gms
2
1
0
31
100BK
32
2mg
acidified
kidney
control
33
10mg
acid
kidney
+
15mg
DPG
5min
34
ditto
10min
35
100BK
36
100BK
37
10mg
acid.
kidney
+
15mg
DPG
15min
38
ditto
20min
39
100BK

if present, may be considered to be insignificant because dose-response curves (FIGURE 8) show that kinin release is linearly related to the concentration of homogenate in the incubate, and this was seen in a variety of renal preparations. Also, yields of kinin from rat renal extracts are equivalent to those reported by Nustad using a semi-purified kininase-free preparation.

b) Preliminary investigation of the kidneys of several species of laboratory animals

Kininogenase activity was detected in renal extracts from cat, rabbit, guinea-pig, mouse, rat and dog.

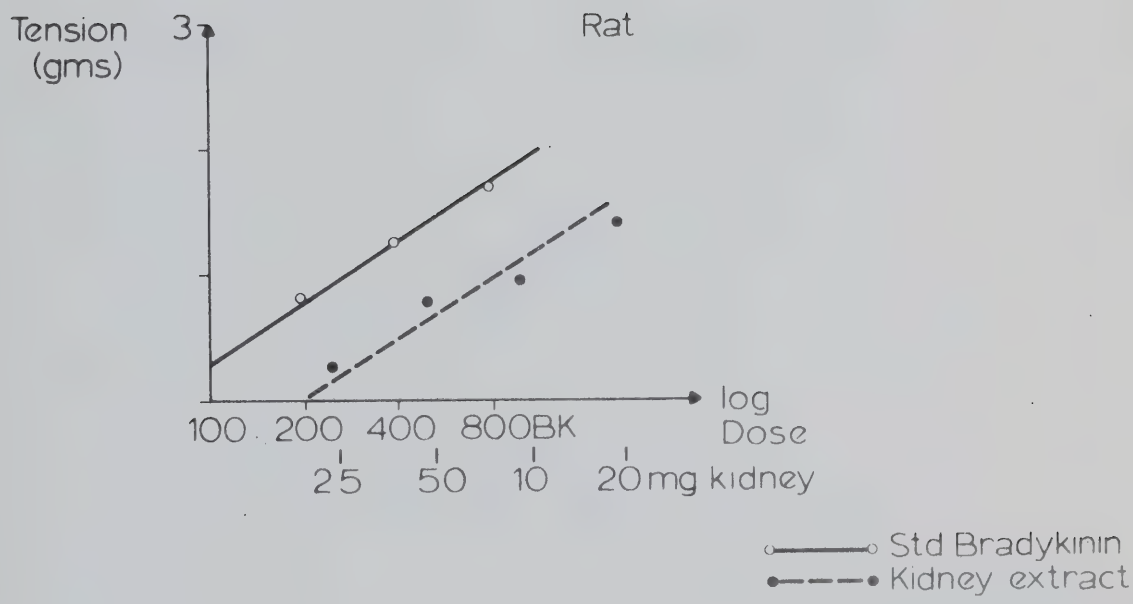
Kidneys were prepared by perfusion and lyophilisation as described previously. The powdered kidney was homogenised in distilled water (20 mg./ml.), acidified to pH 2.0 for 20 minutes at room temperature and the precipitate removed by centrifugation. The pH was then returned to 7.0, and aliquots of the extract incubated with 0.3 ml. of dog pseudoglobulin (50 mg./ml. in isotonic saline) at 37 °C for 20 minutes.

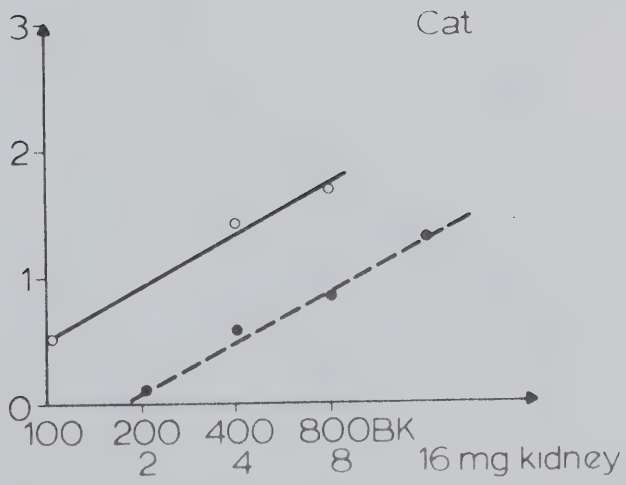
The kinin liberated was compared with concentrations of standard synthetic bradykinin utilising the guinea-pig ileum. Three or more concentrations of bradykinin and the homogenate were utilised giving a range of responses. Dose response curves were then drawn, by plotting response against the logarithm of dose.

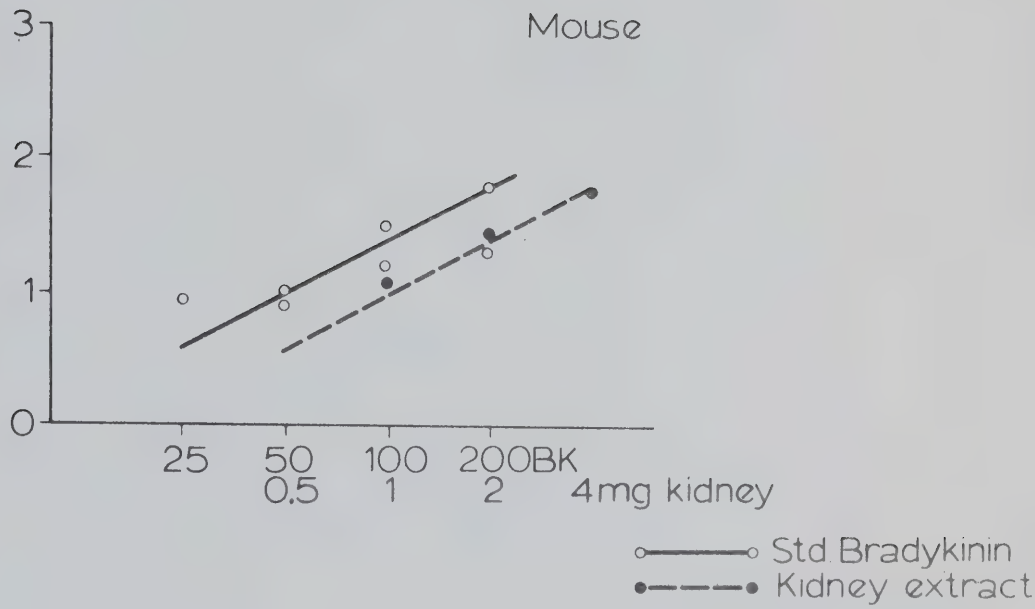
The results are illustrated in FIGURE 8 (a, b and c), and are based upon one assay series in the case of each

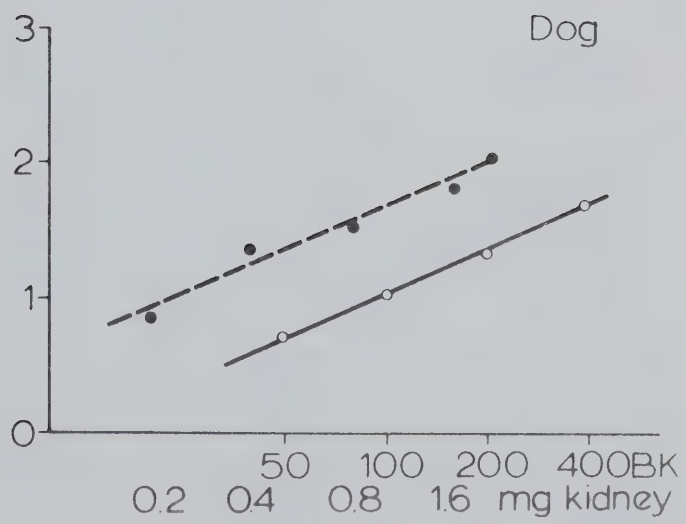
FIGURE 8 (a, b and c)

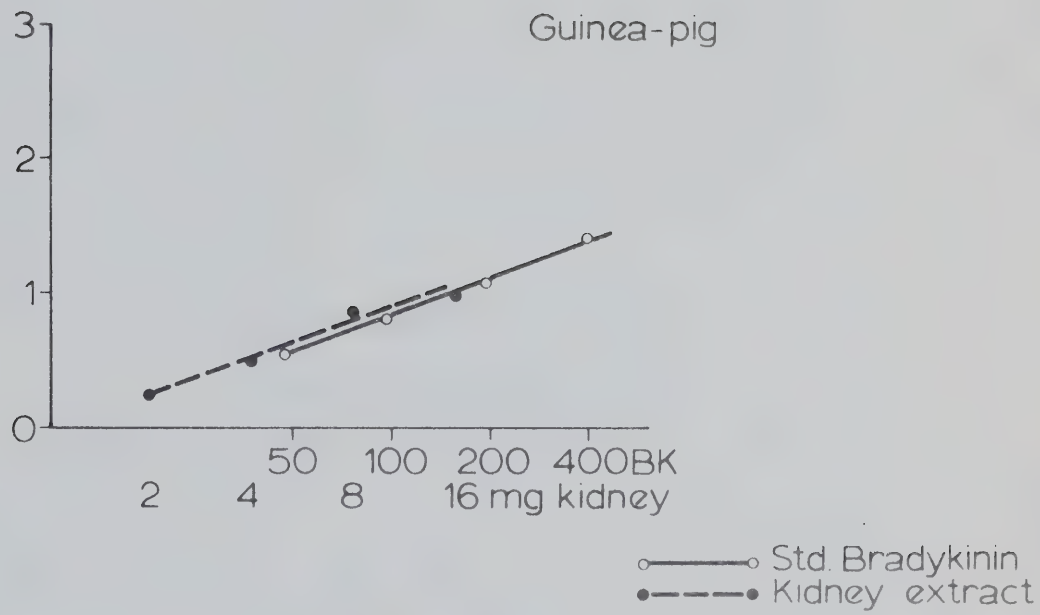
Dose Response curves obtained from renal kininogenase of rat, cat, mouse, dog, guinea-pig and rabbit

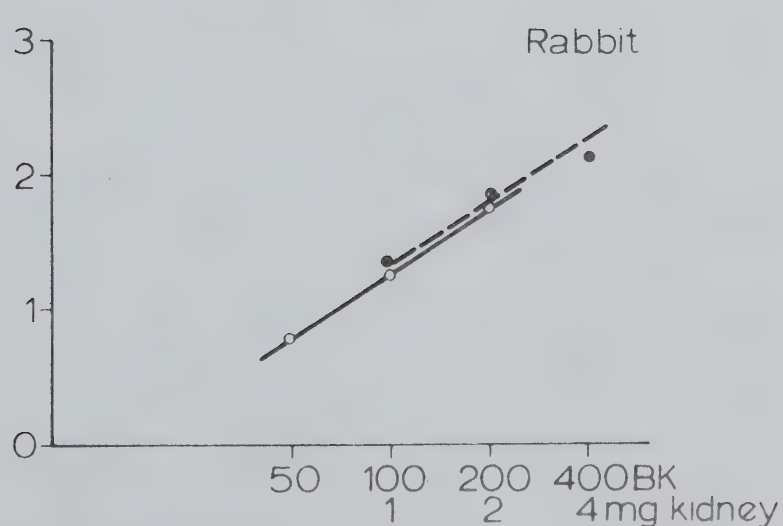

Tension
(gms)
3
Rat
100
200
400
800BK
log
Dose
25
50
10
20 mg kidney
Std Bradykinin
Kidney extract


3
2
1
0
Cat
100
200
2
400
4
800BK
8
16 mg kidney


Mouse
3
2
1
0
25
50
0.5
100
1
200BK
2
4mg kidney
Std. Bradykinin
Kidney extract


Dog
3
2
1
0
50
0.2
100
0.4
200
0.8
400BK
1.6
mg kidney


Guinea-pig
3
2
1
0
50 100 200 400BK
2 4 8 16 mg kidney
Std. Bradykinin
Kidney extract


Rabbit
3
2
1
0
50 100 200 400BK
1 2 4 mg kidney

preparation. From the dose-response curves, an estimate of the activity of the preparations was obtained, and is shown in TABLE 2.

Conclusions concerning the species distribution of renal kininogenase are not possible from these results. It was subsequently shown (Section c, below) that the pH optimum of renal kininogenase is between 8 and 10.5 indicating that these experiments were not performed under optimal conditions. However, the finding that kininogenase was detectable in all cases is significant.

c) Optimal incubation conditions

i. Substrate concentrations

Lyophilised dog pseudoglobulin was dissolved in isotonic saline (10 to 100 mg./ml). Aliquots of an acidified rat renal homogenate equivalent to 10 mg. of lyophilised kidney were incubated with various concentrations of the substrate (pH 7.0, 37°C for 20 minutes) and the kinin liberated was estimated.

It was found that maximum liberation of kinin occurred in the presence of 5 mg. of the substrate. In all subsequent experiments 15 mg. of dog pseudoglobulin substrate was used.

ii. pH optima

Several preparations of kininogenase were compared with respect to their pH optima.

Rat renal kininogenase - Lyophilised renal tissue was prepared as described previously and homogenised in distilled water (10 mg./ml.). The homogenate was acidified to pH 2.0

TABLE 2

Renal Kininogenase of several species

Species	Kininogenase (nanograms of bradykinin equivalent to 1mg. of lyophilised tissue)
Rat	33.3
Cat	23.4
Guinea-pig	11.0
Rabbit	110.0
Mouse	50.0
Dog	400.0

for 20 minutes and then returned to pH 7.0. Aliquots containing between 1 and 2 mg of lyophilised tissue were then incubated with the substrate, over a range of pH values (20 minutes at 37°C).

Dog renal kininogenase - prepared in the same manner as rat kininogenase extract.

Dog urinary kininogenase - dog urine was dialysed against distilled water for 20 hours at 4°C. Aliquots of this solution were then incubated with the substrate.

Dog plasma kallikrein - prepared by the method of Webster and Pierce (1961).

Heparinised whole blood was centrifuged at 12,000 r.p.m. for 20 minutes at 4°C. The supernatant was heated (57°C for 90 minutes) and centrifuged (10,000 r.p.m. for 20 minutes). 100 mls. of the supernatant were then treated with 20 mls. of acetone and the mixture allowed to stand for four hours. The plasma was then dialysed against de-ionised water overnight and the precipitate removed by centrifugation. A portion of the supernatant was diluted to five times its volume with distilled water, and DEAE cellulose added with stirring to reach a final concentration of 200 gms. per litre. The mixture was stirred for one hour and the cellulose removed by filtration. The filtrate was lyophilised.

The lyophilised extract was dissolved in saline (5 mg./ml.) and 1 ml. aliquots of this solution used for incubation and assay.

Duplicate incubations were performed in each case,

and the kinin released plotted against the pH of the incubate. The results are shown in FIGURE 9 a, b and c.

The optimal pH of rat renal kininogenase was found to occur between 8.5 - 11.0. Carvalho (1970) finds the optimal pH between 9 and 10.0, whereas Nustad finds a pH optimum at 8.5 (Nustad 1970 a and b).

Dog renal and urinary kininogenases exhibited pH optima between 8.0 and 10.5 (FIGURE 9 b), while dog plasma kallikrein showed optimal activity between pH 7.0 - 9.0.

The significance of these findings are considered in the DISCUSSION. In all subsequent experiments (Section 2) rat and dog renal kininogenases were incubated with dog pseudoglobulin at pH 9.0.

d) The effect of proteolytic inhibitors

Kininogenases from various sources exhibit differences in sensitivity to proteolytic inhibitors, these differences offering a means of discrimination (APPENDIX).

The differentiation of plasma,urinary and renal kininogenases was attempted, in order to determine whether urinary kininogenase is derived from a renal or plasma source.

Kininogenases prepared as described in subsection c. ii. above were incubated for 10 minutes at 37°C with either isotonic saline (control experiments) or the inhibitor under investigation. Dog pseudoglobulin substrate was then added (15 mg.) and the solutions incubated for 20 minutes at 37°C. The kinin released by the control and inhibitor-containing

FIGURE 9 a

The optimal pH of rat renal kininogenase

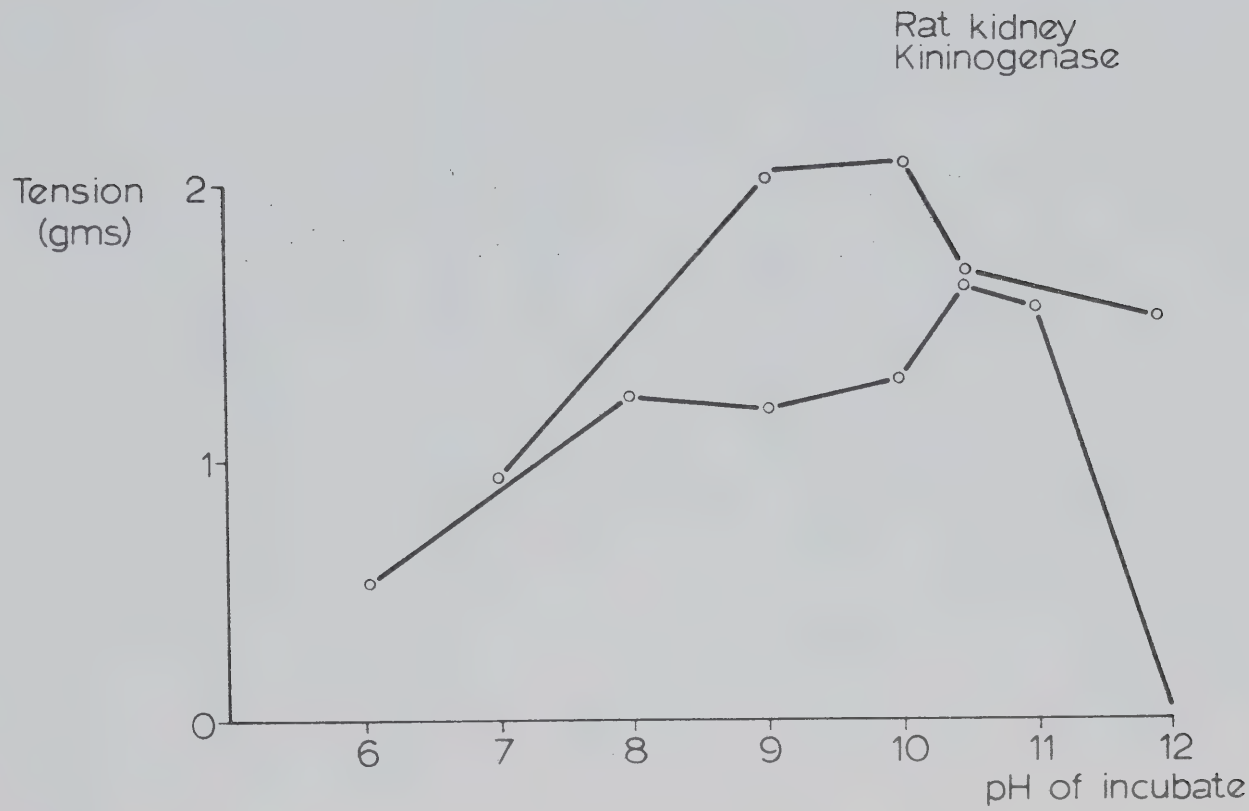
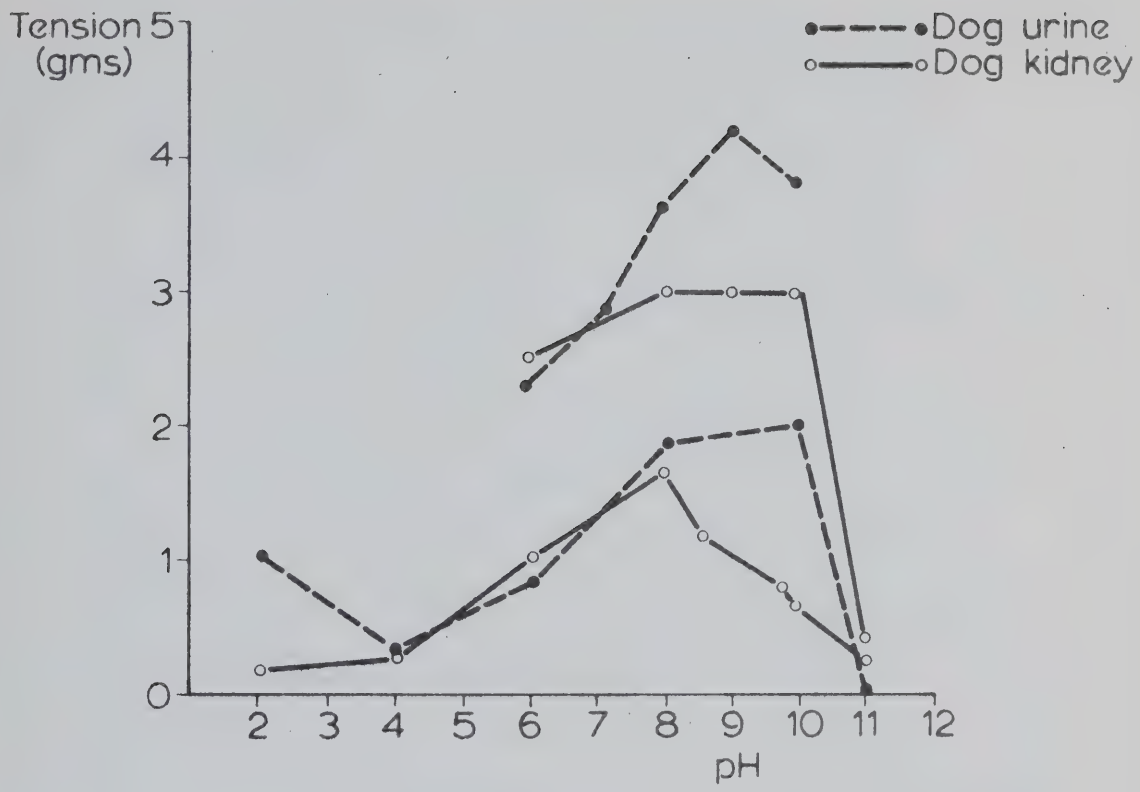

Rat kidney
Kininogenase
Tension
(gms)
2
1
0
6
7
8
9
10
11
12
pH of incubate

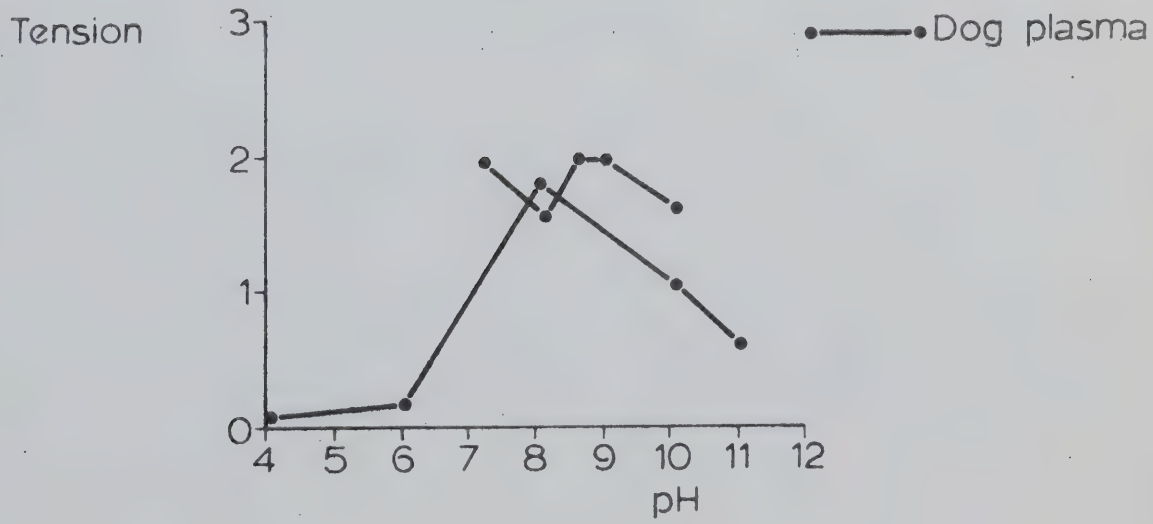
FIGURE 9 b (top)

The optimal pH of dog renal and urinary kininogenase

FIGURE 9 c (bottom)

The optimal pH of dog plasma kallikrein


Tension (gms)
5
4
3
2
1
0
2 3 4 5 6 7 8 9 10 11 12
pH
Dog urine
Dog kidney


Tension
3
2
1
0
4 5 6 7 8 9 10 11 12
pH
Dog plasma

solutions was then compared with concentrations of synthetic bradykinin as described previously.

The effectiveness of the inhibitors against trypsin (10 mg./ml. in isotonic saline) was also tested.

The inhibitors utilised were as follows:

- Trasylol, 500 and 50 units
- Ovomucoid inhibitor (OMI) 0.1, 1, and 5 mg. in isotonic saline
- Soyabean trypsin inhibitor, 0.1, 1, and 5 mg. in isotonic saline.

The results of four experiments are shown in TABLE 3.

The results suggest that dog renal and urinary kininogenase exhibit similarities with respect to the actions of inhibitors. However, they differ from plasma kallikrein, in that they are less sensitive to inhibitors within the dose ranges utilised.

A much larger number of assays with more varied inhibitor concentrations are required before any detailed conclusions can be made. However, evidence from other sources (INTRODUCTION) suggests that urinary and renal kininogenase are similar, and this view is supported by these experiments.

Nustad (1970 a) found that rat renal and urinary kininogenase were similar, both being relatively insensitive to OMI and SBTI. Similar results were reported by Carvalho and Diniz (1966).

Table 3

The Effect of Proteolytic Enzyme Inhibitors

Expt No	Kininogenase	Percentage inhibition of kinin release: Trasylol 50	Trasylol 500U	OMI 0.1	OMI 1	OMI 5mg	SBTI 0.1	SBTI 1	SBTI 5mg.
	Trypsin								
1	0.1 mg	87		24			50		
2	1.0 mg		100		92			100	
3	1.0 mg		71			93			63
4	1.0 mg		72			52			100
	Dog plasma kallikrein								
1	0.1 mg	60		74			60		
3	1.0 mg		38		63			13	
4	2.5 mg		60			0			100
	Dog renal kininogenase								
2	1.0 mg		0		0			13	
3	0.1 mg		31			48			13
4	1.0 mg		0			11			61
	Dog urinary kininogenase								
2	0.1 mg		0		0			14	
3	0.1 mg		0			10			7
	Rat renal kininogenase								
3	0.5 mg		42			50			50

2. The Distribution of Kininogenase in the Rat Kidney, Cortex-Medulla Separation

The kidneys from six rats were prepared as described under METHODS. The kininogenase activities of the cortex and medulla were calculated and are shown in TABLE 4.

The average kininogenase activity of the cortex was 134 ± 27.9 ng. of synthetic bradykinin/mg. of protein, compared to the medulla which contained 36.7 ± 9.4 ng. of bradykinin/ml. of protein. This distribution is graphically illustrated in FIGURE 10.

It was found that the cortex represented approximately 80% of the total kidney dry weight in the case of the six kidneys examined in TABLE 5. From the total dry weight and the kininogenase activity per mg. of dry tissue, it was possible to determine the total kininogenase content of the cortex and medulla, and then express this as a percentage of the total kidney kininogenase. It was found that the cortex contained 92.0 ± 1.5% and the medulla 8.1 ± 1.5%. These results are shown in TABLE 6 and graphically presented in FIGURE 11.

The results demonstrate that kininogenase is primarily localised within the renal cortex, this finding being in agreement with that of Nustad (1970 b). Nustad expresses his results in different units, however, he provides sufficient information to extrapolate and compare them to this study. Kininogenase activity was expressed as micrograms of bradykinin liberated per gm. wet weight of renal cortex after one minute of incubation with substrate. Assuming a constant rate of

TABLE 4

RAT -------- CORTEX, MEDULLA SEPARATIONS 1969-70

Rat No	Kidney Sample	ng Bradykinin liberated by lmg lyophilised tissue	Protein (ug bovine albumin) equivalent to lmg lyophilised tissue	ng bradykinin per mg of protein
1	Left cortex	92.5	240	385.0
	left medulla	28.0	430	65.2
	Right cortex	51.0	385	132.5
	right medulla	28.3	345	82.1
2	Left cortex	66.8	315	212.0
	left medulla	14.4	555	25.9
	Right cortex	30.0	410	73.2
	right medulla	2.3	640	3.6
3	Left cortex	33.0	485	68.2
	left medulla	4.0	720	5.6
	Right cortex	12.3	460	26.8
	right medulla	7.3	555	13.2
4	Left cortex	44.6	388	112.5
	left medulla	7.9	684	11.6
	Right cortex	36.6	425	86.5
	right medulla	7.2	302	23.8
5	Left cortex	70.8	393	182.0
	left medulla	31.6	389	81.3
	Right cortex	51.3	284	180.1
	right medulla	25.1	284	88.3
6	Left cortex	22.5	311	72.5
	left medulla	5.0	315	16.5
	Right cortex	23.7	311	76.4
	right medulla	7.6	324	23.4

Key: ug - microgram (10^{-6}gm)
ng - nanogram (10^{-9}gm)

FIGURE 10: Rat, cortex medulla
kininogenase distribution

RAT: CORTEX-MEDULLA KININOGENASE DISTRIBUTION

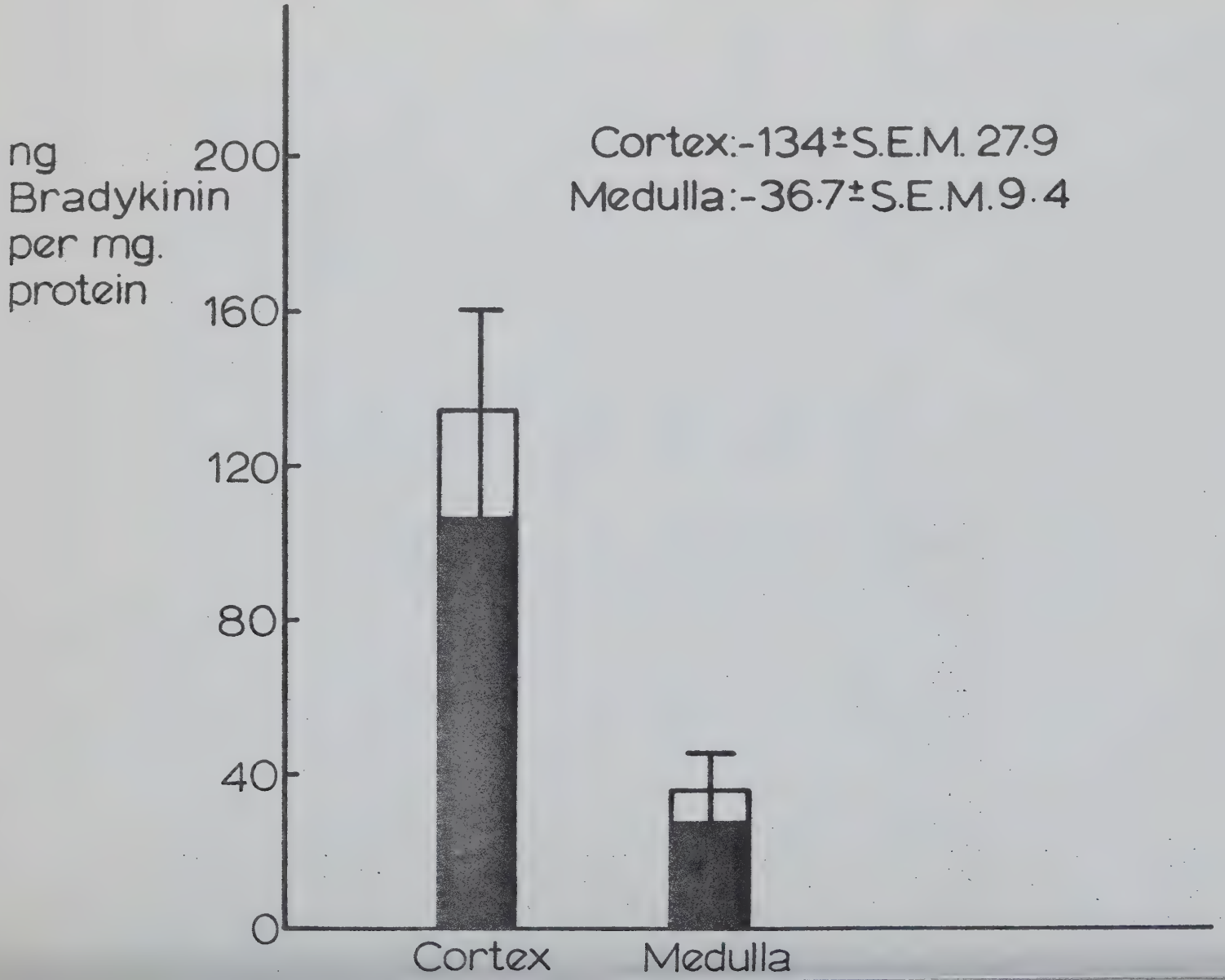


TABLE 5. Percentage composition of rat kidney

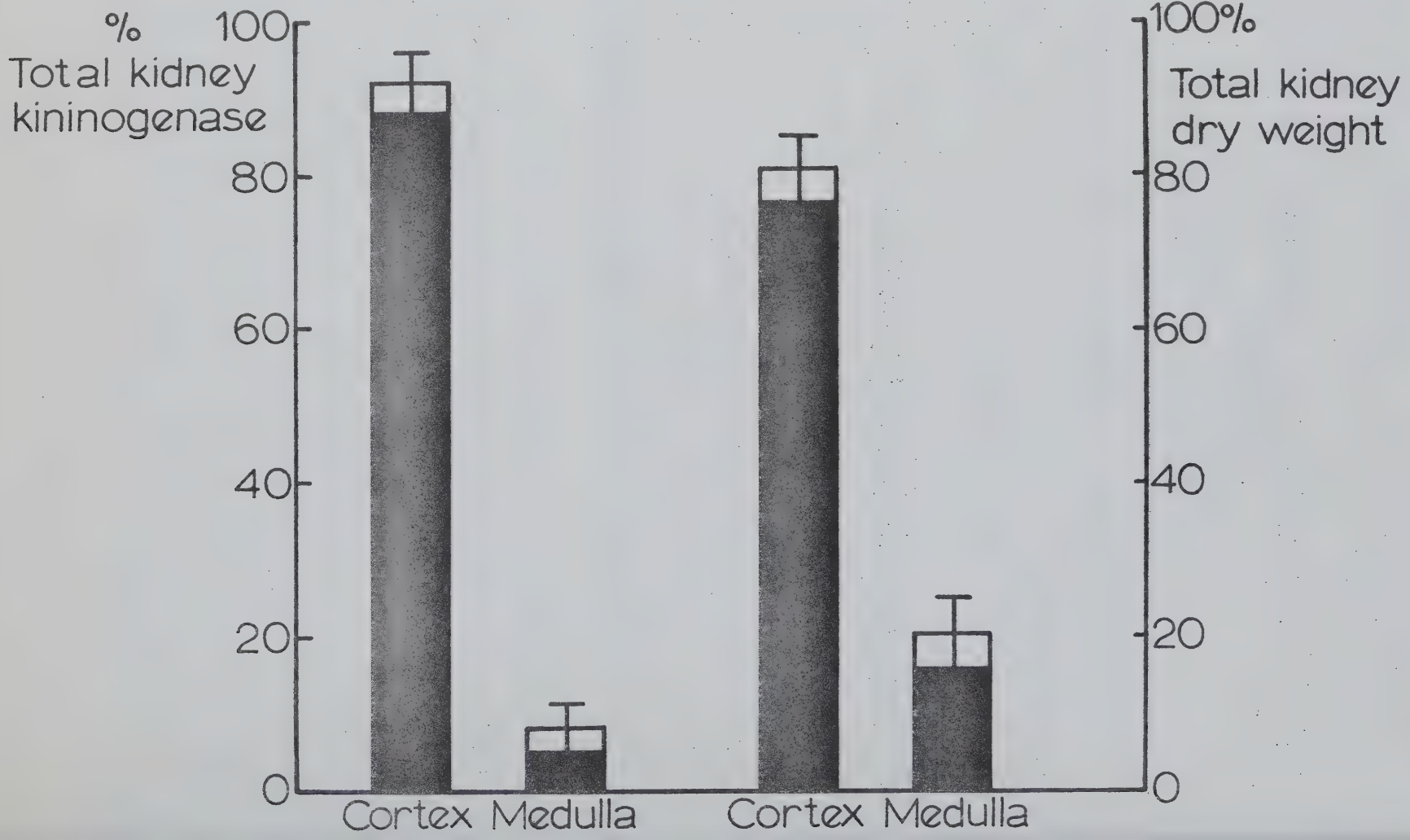
Rat Number	Kidney	Sample	Sample Dry Wt. (mg.)	Total kidney Dry weight (Cortex & medulla)	% total weight
4	Left	Cortex	364.4	445.5	82
		Medulla	81.1		18
	Right	Cortex	363.7	428.5	85
		Medulla	64.8		15
5	Left	Cortex	437.7	534.1	82
		Medulla	96.4		18
	Right	Cortex	419.7	535.7	78
		Medulla	116.0		22
6	Left	Cortex	303.6	411.2	74
		Medulla	107.6		26
	Right	Cortex	330.7	439.4	75
		Medulla	108.7		25

Table 6. Percentage Kininogenase Content of Rat Kidney

Rat Number	Sample		Kininogenase per mg sample (ng Bradykinin) equiv.)	Total Sample Kininogenase (ug Bradykinin)	Total Kidney Kininogenase (cortex & medulla)	% Total Kininogenase in sample
4	Left	Cortex	44.6	16.6	17.2	96.5
		Medulla	7.9	0.6		3.5
	Right	Cortex	36.6	13.3	13.8	96.4
		Medulla	7.2	0.5		3.6
5	Left	Cortex	70.8	31.0	34.1	91.0
		Medulla	31.6	3.0		9.0
	Right	Cortex	51.3	21.5	24.4	88.0
		Medulla	25.1	2.9		12.0
6	Left	Cortex	22.5	6.8	7.7	88.5
		Medulla	5.0	.9		11.5
	Right	Cortex	23.7	7.8	8.6	91.0
		Medulla	7.6	.8		9.0

FIGURE 11: Rat, cortex medulla kininogenase distribution, percentage kidney kininogenase

RAT-CORTEX-MEDULLA KININOGENASE DISTRIBUTION



kinin liberation when extending the incubation period hypothetically to 20 minutes, and utilizing the results of protein determinations, Nustad's findings become:

Cortex - 422 ng. bradykinin per mg. protein

Medulla - 53 ng. bradykinin per mg. protein

This procedure is not entirely justified, as it is unlikely that the same rate of kinin liberation would be maintained over a longer period. However, such a calculation does indicate that enzyme activities within the same order of magnitude were detected in these studies.

3. The Distribution of Kininogenase in the Dog Kidney

Glomerular, tubular and medullary fractions were prepared from thirteen dog kidneys by the procedure described under METHODS. The first seven kidneys were utilized as a pilot study to determine technical problems. The main difficulty that arose was a low yield of glomerular tissue. This was corrected in the second series of experiments by re-applying the isolated glomerular and tubular tissues to the separation column at least two times. In this way, glomerular tissue was recovered from the tubular fraction and the purity of both increased.

a) Kininogenase determinations

i. Pilot study

Kininogenase was determined by the procedure described under METHODS, and the results are shown in TABLE 7. The average concentration of kininogenase in each fraction was

TABLE 7. Kininogenase Determinations from

Dog Glomeruli-Tubule Separation, Series 1, 1969

Kidney No.	Extract	Extract concentration mg/ml	Bradykinin (ng) equiv. to 1mg tissue	Protein (ug) equivalent to 1mg tissue	Bradykinin (ug) per mg protein
1	Glom	50	8.5	6.0	1.46
	Tub	40	26.5	85.0	0.32
2	Glom	50	14.8	14.0	1.06
	Tub	40	40.0	2.5	16.00
	Med	20	17.2	360.0	0.48
3	Glom	50	14.9	77.0	0.19
	Tub	40	14.3	235.0	0.06
	Med	20	21.0	380.0	0.05
4	Glom	20	54.4	44.7	1.22
	Tub	20	75.2	40.5	1.85
	Med	20	7.1	120.2	0.06

5	Glom	20	34.2	27.4	1.24
	Tub	20	137.2	61.8	2.21
	Med	50	12.7	-	-
6	Glom	50	29.5	11.8	2.50
	Tub	50	31.5	24.4	1.29
	Med	50	17.5	60.9	0.29
7	Glom	50	14.5	78.4	0.19
	Tub	50	50.3	74.4	0.68
	Med	50	15.5	170.0	0.09

found to be; glomeruli 1.12 ± 0.3, tubules 3.20 ± 2.15 and medulla 0.19 ± 0.08 micrograms of bradykinin per milligram of protein. The distribution of kininogenase is shown in FIGURE 12. Considerable variability was found in the tubular extract, and considering the low yield of the glomerular fractions, it was decided to repeat the experiments.

ii. The second series of investigations

The yields of tissue obtained from three experiments are shown in TABLE 8. Unfortunately the data from the entire series of investigations is no longer available.

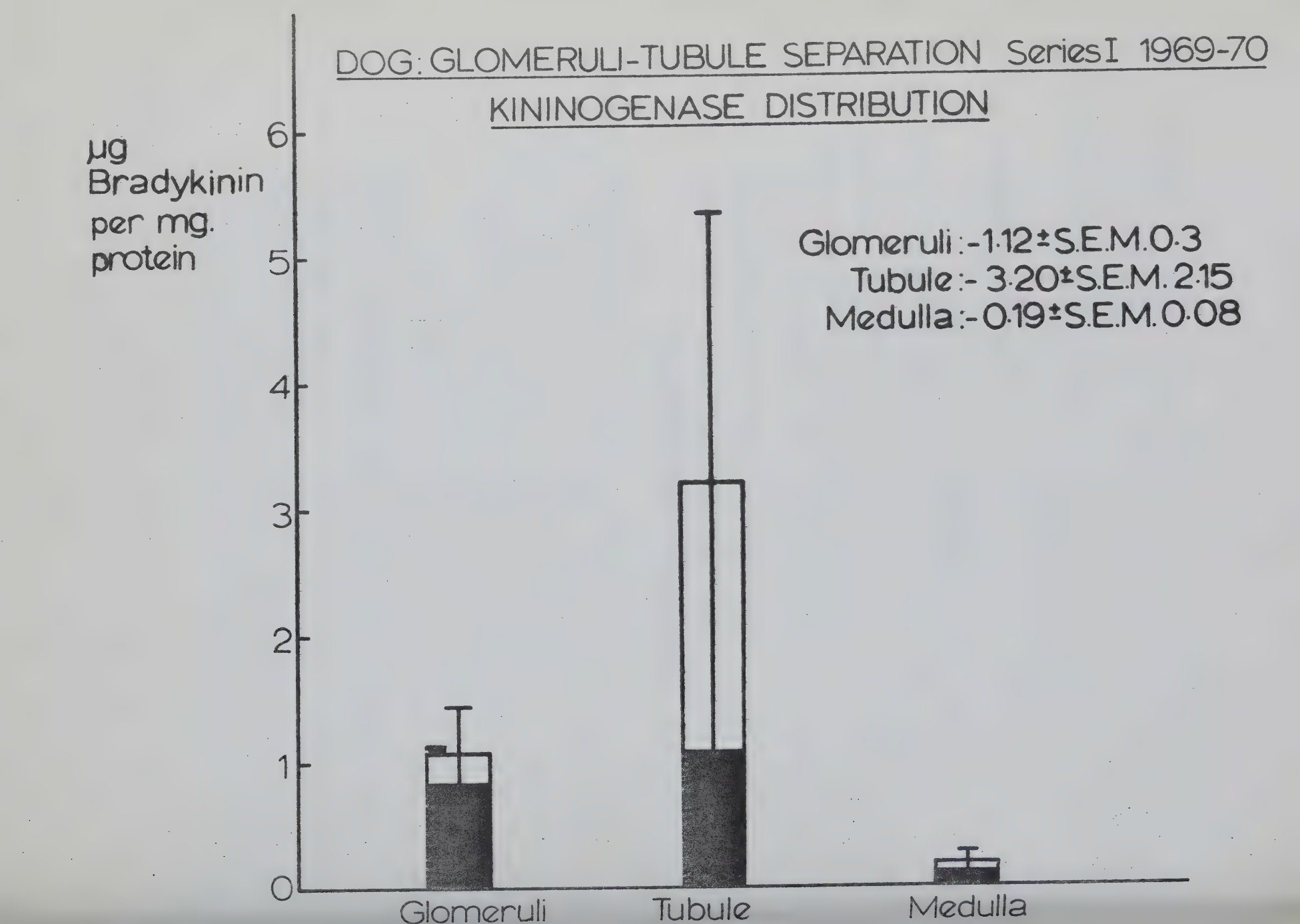
TABLE 8. Yields of Dog Renal Fractions

		Lyophilised tissue weight (mg.)		
Dog No	Kidney	Glomeruli**	Tubule	Medulla
1*	Left	144.0	750.0	995.9
	Right	268.8	519.9	735.1
2	Left	63.9	434.9	n/a
	Right	354.3	606.7	1,576.1
4	Left	108.2	1,007.0	1,331.6
	Right	119.5	n/a	1,382.1

* this particular series of samples were discarded due to an error in extract preparation.

** indicates weight of glomerular tissue containing iron oxide particles.

FIGURE 12: Dog glomeruli-tubule separation series I, 1969-70 kininogenase distribution


DOG: GLOMERULI-TUBULE SEPARATION Series I 1969-70
KININOGENASE DISTRIBUTION
µg Bradykinin per mg. protein
6
5
4
3
2
1
0
Glomeruli :- 1·12 ± S.E.M. 0·3
Tubule :- 3·20 ± S.E.M. 2·15
Medulla :- 0·19 ± S.E.M. 0·08
Glomeruli
Tubule
Medulla

The concentration of kininogenase in the fractions obtained from six kidneys was determined, the increased yield of glomerular tissue permitting duplicate assays in most cases. The assay procedure is illustrated in FIGURE 13. Three concentrations of each extract were incubated with the substrate and the kinin released was then compared to three concentrations of synthetic bradykinin using the guinea-pig ileum. Dose response curves were drawn, and from these the activity of the fraction was determined.

The results are shown in TABLE 9 and FIGURE 14. The average kininogenase activity of the three fractions was; glomeruli 10.42 $\pm$ 2.37, tubules 3.24 $\pm$ 0.5 and the medulla 0.58 $\pm$ 0.09 micrograms of bradykinin per milligram of protein.

b) Histological investigations

Each kidney investigated in the second series of experiments was subjected to histological examination at two stages in the preparation of renal fractions. A sample of the renal cortex was taken to show the effectiveness of perfusion with iron particles, and samples of the final fractions were taken to determine their purity. The procedure was described under METHODS.

FIGURE 15, is a collection of microphotographs illustrating typical findings.

FIGURE 15 a and b shows the penetration of iron oxide particles into the renal cortex, and black deposits are seen to be associated with the major blood vessels and the capillary

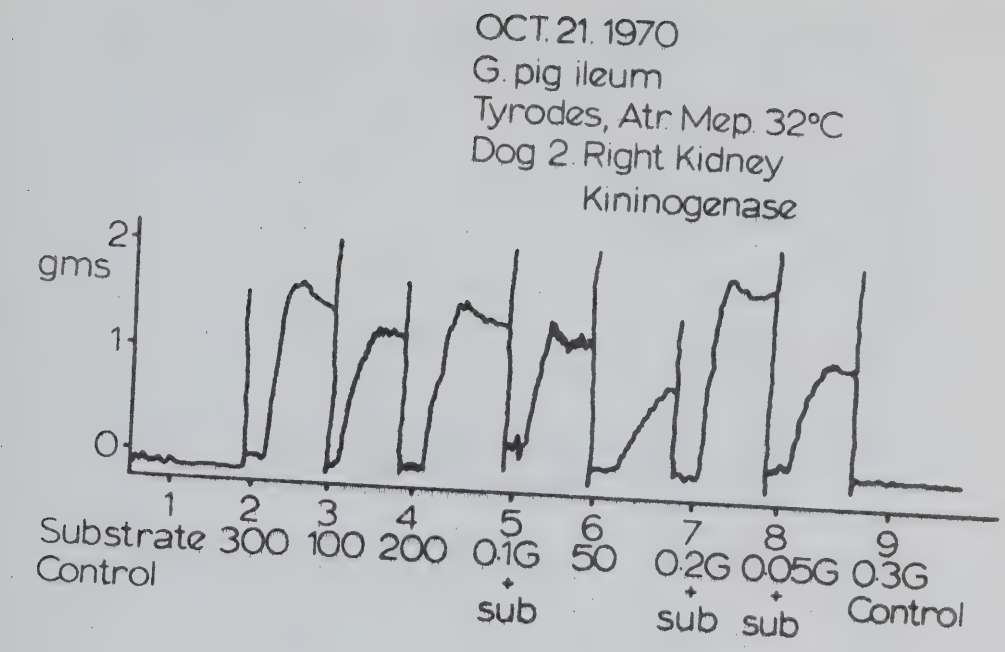
FIGURE 13. The Assay of Kinin Liberated by Dog Renal Fractions

Key: 300,200 and 100 are nanogram concentrations of synthetic bradykinin.

- substrate control, is an incubate of dog pseudoglobulin with isotonic saline
- 0.3 G control is an incubate of 0.3 mls. of the glomerular extract with isotonic saline.
- G. glomerular fraction
- T. tubular fraction
- M. medullary fraction

Incubation conditions

20 mins at 37°C, pH 9.0.


OCT. 21. 1970
G. pig ileum
Tyrodes, Atr. Mep. 32°C
Dog 2. Right Kidney
Kininogenase
gms
2
1
0
1 2 3 4 5 6 7 8 9
Substrate 300 100 200 0.1G 50 0.2G 0.05G 0.3G
Control
+ sub
+ sub
+ sub
Control

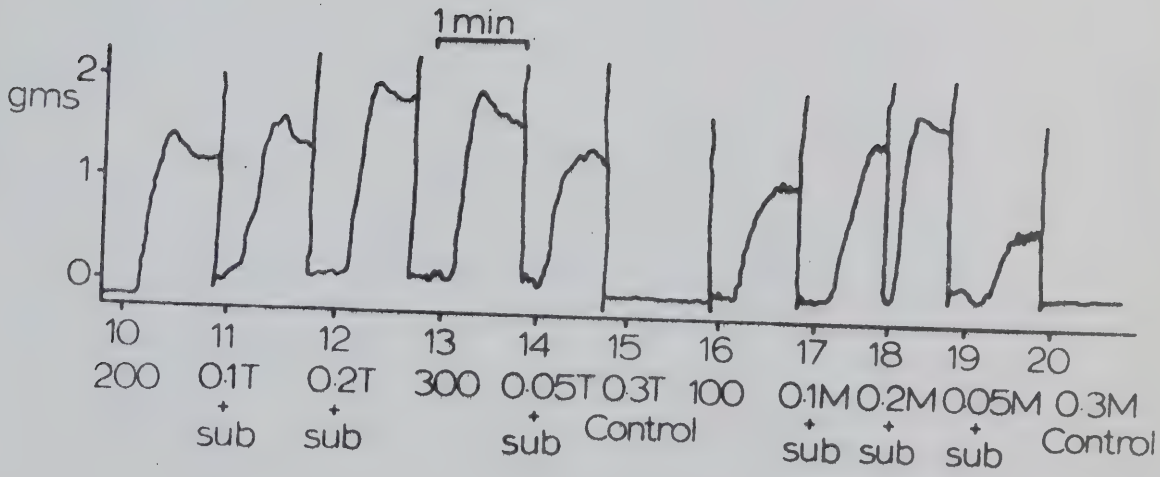

1 min
gms
2
1
0
10 11 12 13 14 15 16 17 18 19 20
200 0.1T 0.2T 300 0.05T 0.3T 100 0.1M 0.2M 0.05M 0.3M
+ sub
+ sub
+ sub
Control
+ sub
+ sub
+ sub
Control

TABLE 9. Kinogenase Determinations From

Dog: Glomeruli-Tubule Separation, Series II 1970-71

Sample	Extract concentration mg/ml	Protein concentration ug/ml	Bradykinin (nanograms) equivalent to 1ml extract	Bradykinin (micrograms) equivalent to 1 mg protein
2RG	25	224	1935	8.63
			1442	6.43
		425	1133	2.66
			1100	2.59
			2550	6.00
2RT	25	317	1735	5.48
			1520	4.78
		400	1870	4.67
			2600	6.50
2RM	50	1172	1400	1.19
			543	0.46
		945	532	0.56
			333	0.35
2LG	-	-	-	-
2LT	25	545	1532	2.81
			3067	5.64
2LM	50	980	337	0.34
			600	0.61

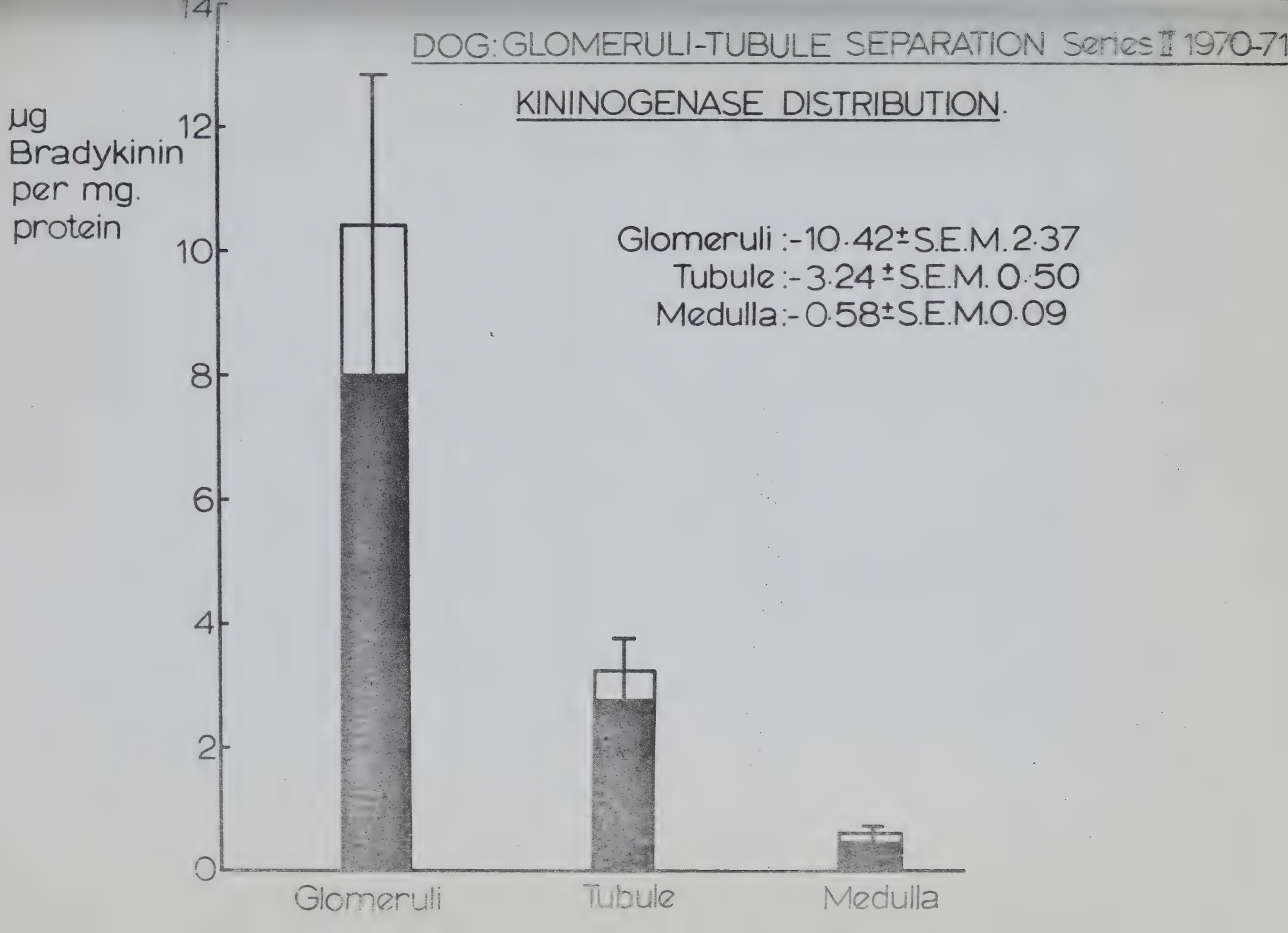
3RG	25	85	2270	26.70
			2000	23.60
			1600	18.90
3RT	25	511	1935	3.40
			1065	2.10
			1200	2.40
3RM	50	533	350	0.70
			87	0.16
3LG	25	130	900	6.92
			1600	12.30
3LT	25	855	466	0.55
			1033	1.21
			1000	1.17
3LM	50	785	266	0.34
			600	0.77
4RG	25	227	1265	5.60
4RT	25	585	1665	2.80
4LG	25	281	1333	4.74
4LT	25	867	1665	1.92
4LM	50	453	426	0.94

Key: 2RG - denotes dog 2, right kidney, glomerular fraction
2RT - " " " " " tubular fraction
2RM - " " " " " medullary fraction

FIGURE 14: Dog glomeruli-tubule separations, series II, 1970-71 kininogenase distribution

DOG: GLOMERULI-TUBULE SEPARATION Series II 1970-71

KININOGENASE DISTRIBUTION.



Glomeruli :- 10·42 ± S.E.M. 2·37
Tubule :- 3·24 ± S.E.M. 0·50
Medulla :- 0·58 ± S.E.M. 0·09

tufts of the glomerulus. There was no evidence to show iron particles in the tubular tissues of any of the kidneys investigated.

FIGURE 15 c and d, show an isolated glomerular fraction. Both intact and ruptured glomeruli are seen to be dissociated from other tissues. The vigorous preparatory procedures followed by centrifugation more commonly led to a compressed appearance as seen on the left and right of FIGURE c. Tubular tissue was occasionally observed in each sample, however, this contamination represented only a minor fraction of the total tissue present. Contamination was not measured, although by subjective evaluation, a tubular content of less than 5% was estimated.

FIGURE 15 e shows a tubular fraction. These were always lightly staining and contained very few iron particles. As with the glomerular fraction, slight contamination was occasionally observed, but was considered to be insignificant.

FIGURES 15 f and g illustrate the glomerular and tubular fractions from another kidney, the appearance is similar.

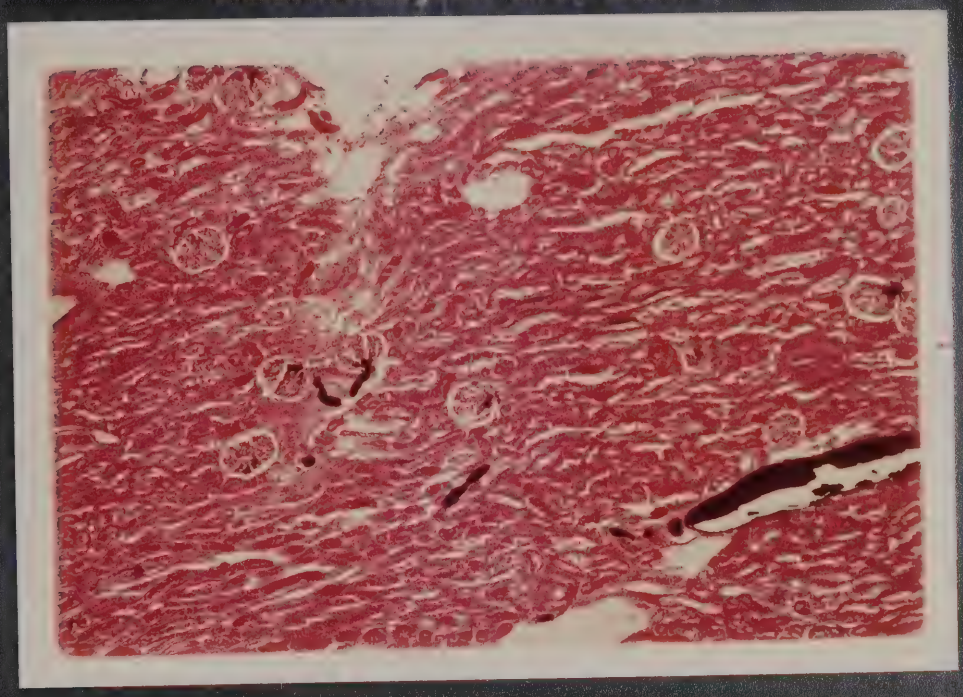
c) Qualitative experiments

There are many substances extractable from renal tissues that may stimulate smooth muscle preparations such as the guinea-pig ileum. These include prostaglandins, acetylcholine, epinephrine, histamine and serotonin. These substances do not require incubation with a substrate to exert their effects, however, it was essential to demonstrate that such

Figure 15

Iron Perfused Renal Cortex

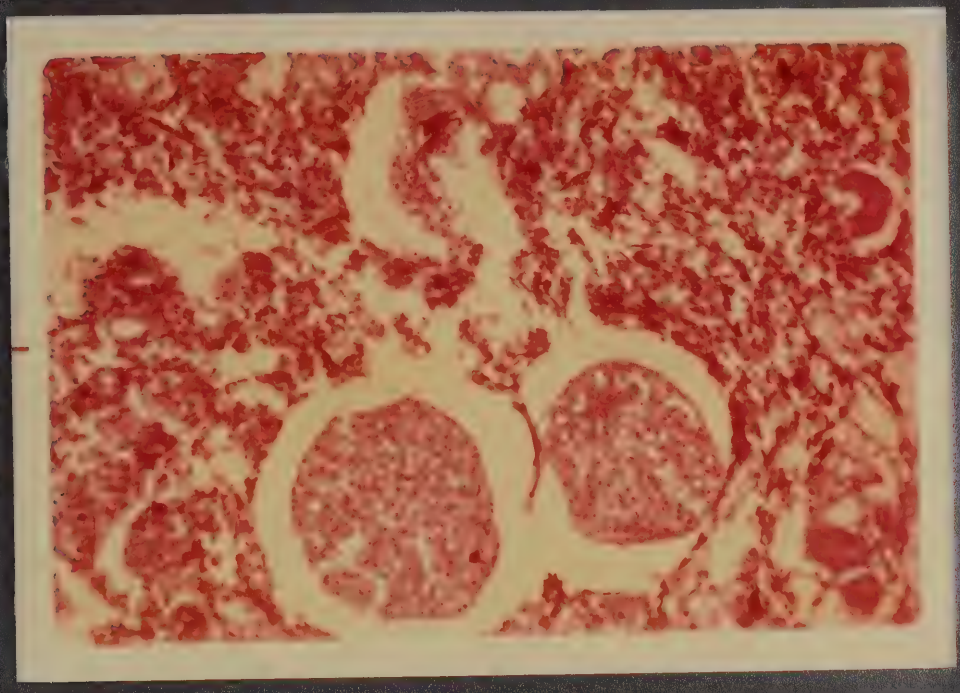
Haematoxylin and Eosin



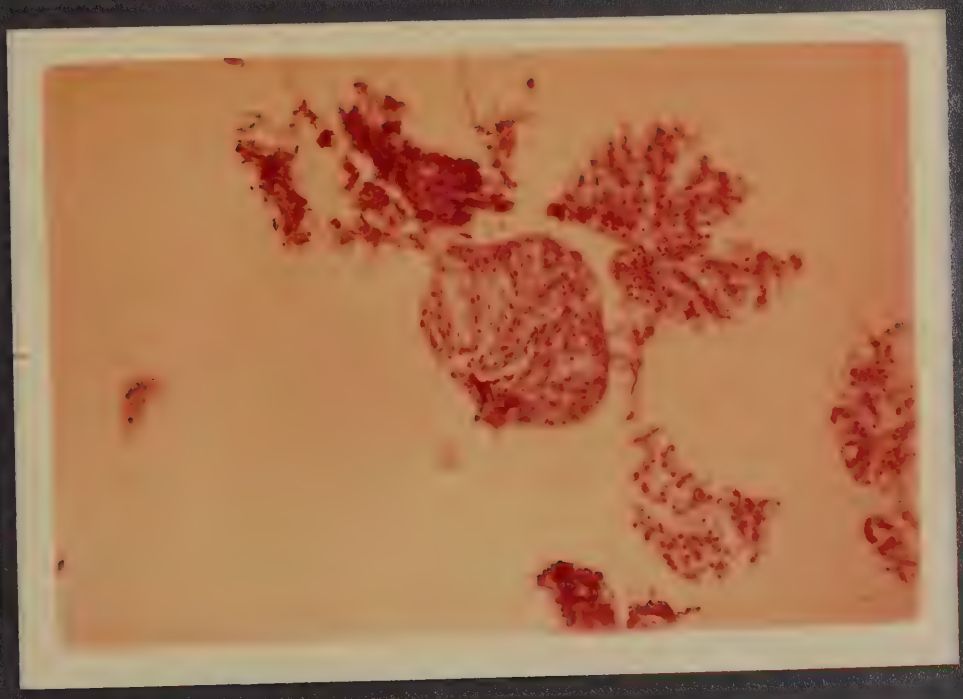
a. Dog 3. Right Kidney x 11 magnification

b. Dog 3. Right Kidney x 70 magnification

Isolated Glomerular Fraction



c. Dog 3. Left Kidney x 70



d. Dog 3. Left Kidney x 70

Isolated Tubular Fraction

e. Dog 3. Left Kidney x 70

f. Isolated Glomerular Fraction
Dog 2. Right Kidney x 70

g. Isolated Tubular Fraction
Dog 2. Right Kidney x 70

substances were not present in the extracts or substrate used in this study.

This was investigated by incubating aliquots of the renal fractions, and also the substrate with isotonic saline. A typical result is seen in FIGURE 13, where it is shown that control incubates are without activity on the guinea-pig ileum preparation. This procedure was repeated during all experiments and the same results obtained.

i. Experiments with the rat duodenum

Renin, another vasoactive substance found in the kidney, liberates angiotensin from the alpha-2-globulin fraction of the plasma proteins. Angiotensin, like bradykinin stimulates the guinea-pig ileum to contract (DISCUSSION). It was thus considered important to determine whether the activity previously assumed to be kininogenase, was in part due to the presence of contamination with renin. It would not be possible to differentiate the products of these enzyme activities on the basis of experiments with the guinea-pig ileum.

The isolated rat duodenum was utilized in this study. The response to synthetic bradykinin and angiotensin was elicited as shown in FIGURE 16. It is seen that bradykinin leads to a marked relaxation of the preparation, whereas angiotensin commonly produces a contraction (this effect is more clearly shown in FIGURE 20).

Kininogenase extracts prepared from the three dog renal fractions were incubated with the substrate and a

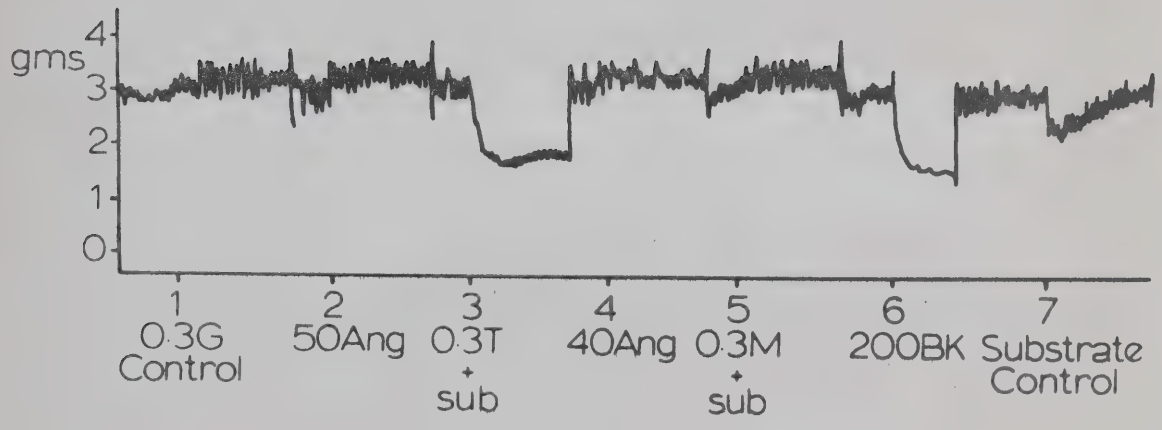
FIGURE 16. The Effects of Dog Renal Kininogenase Incubates upon the Rat Duodenum

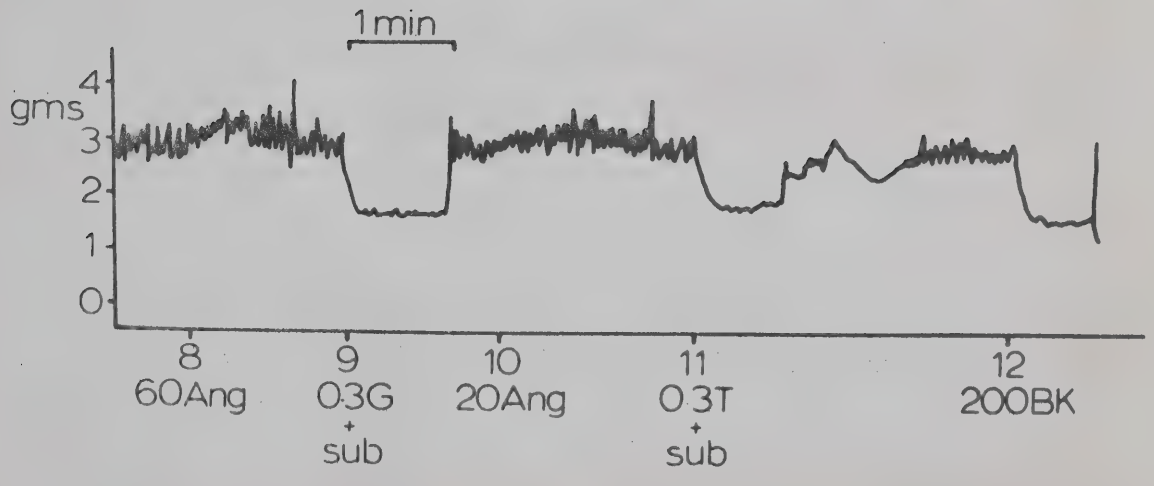
Key: - Ang. angiotensin (nanograms)
- BK. bradykinin (nanograms)
- G. glomerular fraction
- T. tubular fraction
- M. medullary fraction

Incubation conditions

20 mins. at 37°C. pH 9.0.

OCT. 28.1970
Rat duodenum
De Jalons Soln. 31°C
Dog 2. Right Kidney
Kininogenase





marked relaxation of the duodenum was observed in all the experiments. Every kidney extract was tested in this manner. This is presented as evidence to show that the activity present in the kininogenase extracts investigated in this study liberated a kinin after incubation with dog pseudoglobulin substrate.

ii. Experiments with carboxypeptidases A and B

"Carboxypeptidase A has preferential action on peptides containing carboxyl terminal aromatic amino acids and will not split off carboxyl terminal arginine or lysine. Carboxypeptidase B on the other hand has specific preference for splitting off terminal lysine and arginine." (Smyth 1968)

N-arg-pro-pro-gly-phe-ser-pro-phe-arg-C

bradykinin

N-asp-arg-val-tyr-ileu-his-pro-phe-C

val^5 angiotensin II

The structures of the two peptides suggested that carboxypeptidases A and B might selectively inactivate angiotensin and bradykinin. This possibility was investigated as an alternative means of discrimination between these peptides.

Carboxypeptidases A and B (COA and COB, Worthington Biochemical Corp.) were dissolved in isotonic saline to produce concentrations of 100 micrograms and 10 micrograms per ml., respectively.

4 micrograms of carboxypeptidase B were incubated with concentrations of synthetic bradykinin, angiotensin and

also incubates prepared for kininogenase determination. The incubation was carried out for 20 minutes at 37°C. The results are shown in FIGURE 17. It is seen that carboxypeptidase B inactivates bradykinin and also the activity liberated by the kininogenase incubate, whereas angiotensin was unaffected.

40 micrograms of carboxypeptidase A were then incubated with similar solutions under the same conditions. It was found that only angiotensin was affected by this procedure.

These results provide further evidence that the activity liberated from dog pseudoglobulin substrate by dog renal kininogenase was typical of a kinin. There was no evidence for the presence of the renin-angiotensin system.

4. The Distribution of Renin in the Dog Kidney

The procedure for the isolation of glomeruli by the infusion of magnetic particles, was devised for the investigation of renin in the rabbit kidney by Cook and Pickering (1958).

The yield of glomerular tissues obtained from the dog kidneys used in the second series of kininogenase experiments, provided sufficient material for renin determinations by the procedure described under METHODS.

Renin was determined by the incubation of renal extracts with dog renin substrate, followed by the bio-assay of liberated angiotensin using the guinea-pig ileum.

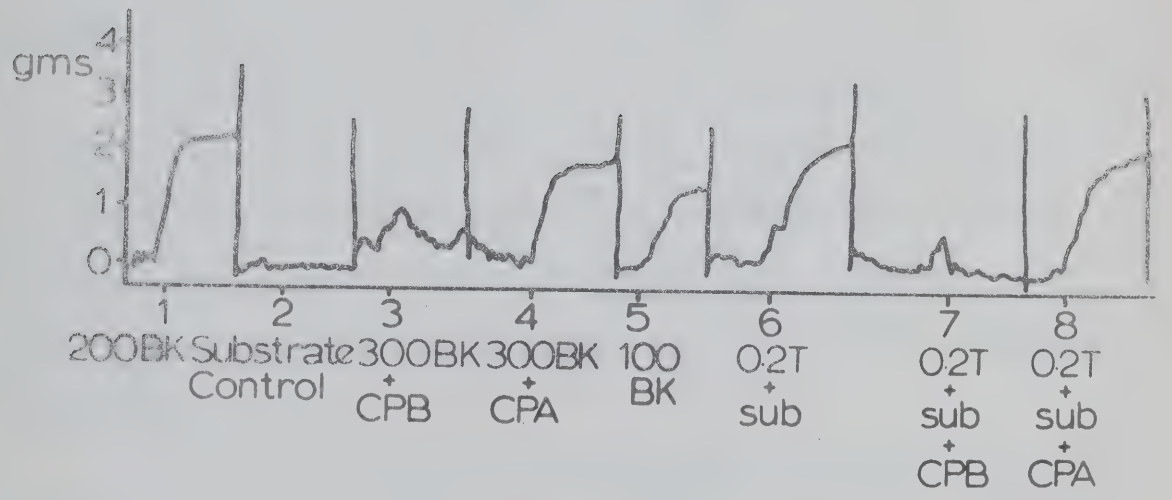
FIGURE 17: The effect of carboxypeptidases A and B on incubates of dog renal kininogenase

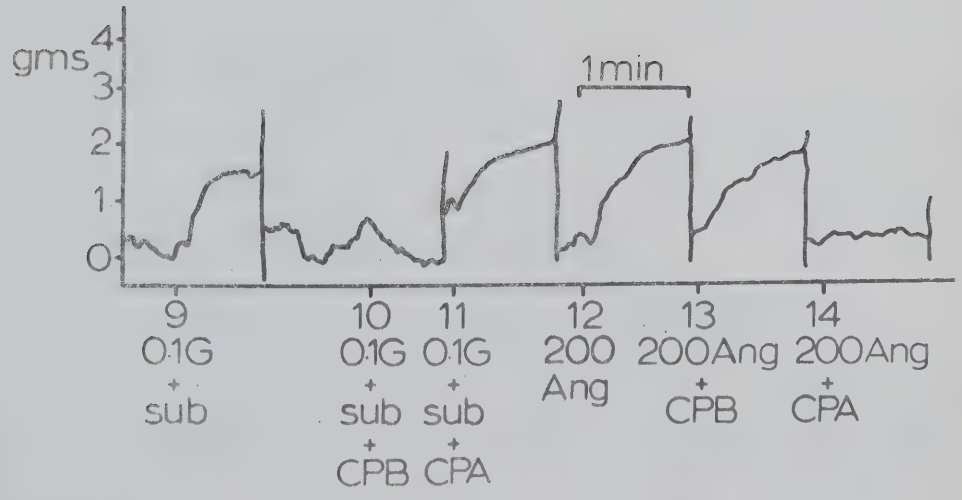
Key:
- Ang. angiotensin (nanograms)
- B.K. bradykinin (nanograms)
- CPA. carboxypeptidase A
- CPB. carboxypeptidase B
- G - glomerular fraction
- T - tubular fraction
- M - medullary fraction

Incubation conditions

20 mins at 37°C. pH 7.0

OCT. 22. 1970
G. pig ileum
Tyrodes, Atr. Mep. 32°C
Dog 2. Right Kidney
Kininogenase





Angiotensin is more commonly assayed by its effects upon the blood pressure of the nephrectomised rat, or more recently by radioimmunoassay. The procedure described is unconventional, however, it was utilised to provide a method whereby renin and kininogenase could be determined using similar techniques and equipment.

FIGURE 18 illustrates a typical experiment. The guinea-pig ileum responded to both standard concentrations of synthetic angiotensin and also to incubates of renal extracts with renin substrate. From such results, a dose response curve was drawn and the activity of the extracts determined.

The results from five kidneys are shown in TABLE 10, and graphically represented in FIGURE 19. Renin activity was found to be; glomeruli 5.29 $\pm$ 1.16, tubules 2.03 $\pm$ 0.61 and medulla 0.75 $\pm$ 0.31 micrograms of angiotensin II per milligram of protein in the extract.

a) Qualitative experiments

These experiments were carried out as described in Section 3 above, in order to establish the nature of the peptide release by the dog renin extracts.

i. Experiments with the rat duodenum

FIGURE 20 shows a typical experiment. Angiotensin produced a contraction of the duodenum, whereas bradykinin produced relaxation. Renin extracts incubated with the substrate led to a complex effect. Both the glomerular and tubular extracts produced a rapidly occurring contraction

FIGURE 18. The Determination of Renin Activity in Dog Renal Fractions

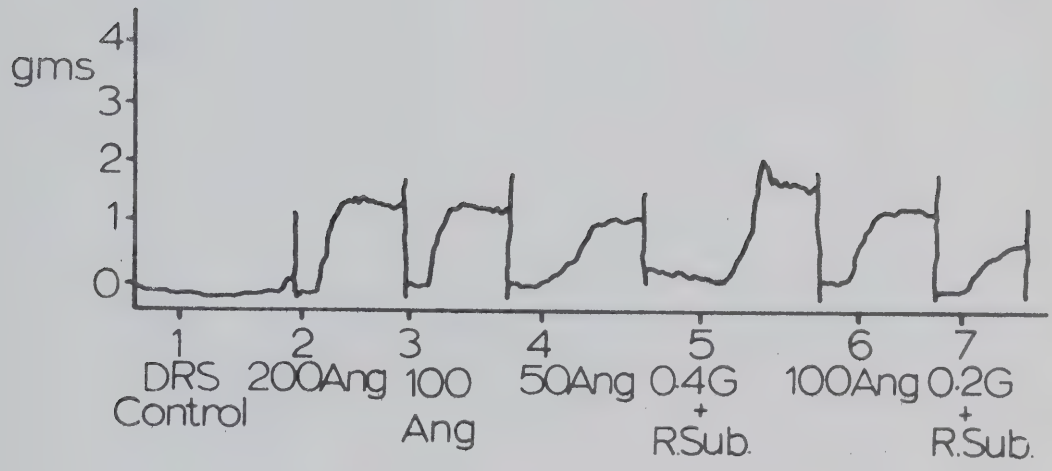
Key: DRS and R.Sub, both refer to dog renin substrate

- Ang. angiotensin (nanograms)
- G. glomerular fraction
- T. tubular fraction
- M. medullary fraction

Incubation conditions:

1 hour at 37°C. pH 5.2

JANUARY 1971
G. pig ileum
Tyrodes. Atr. Mep. 32°C
Dog 2. Right Kidney
Renin



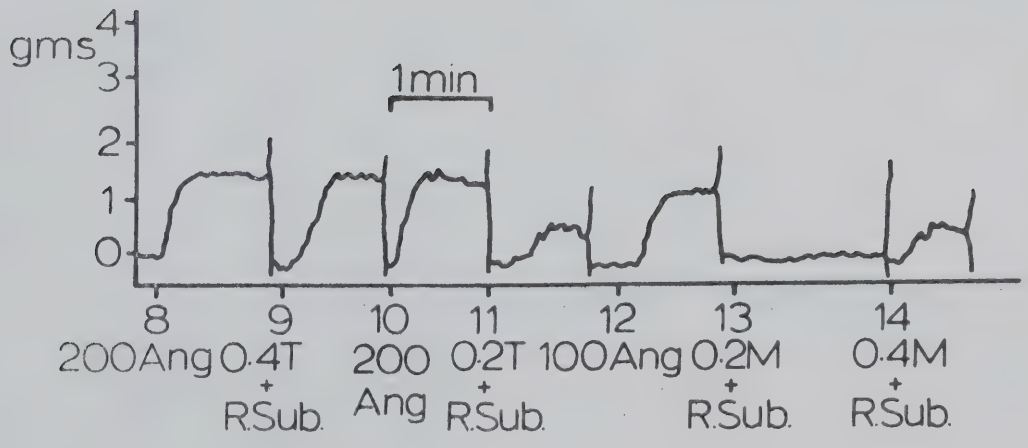


TABLE 10. Dog Renin Determinations

Dog Glomeruli-Tubule Separation, Series II 1970-71

Sample	Extract concentration mg/ml	Protein concentration ug/ml.	Angiotensin II (nanograms) equivalent to 1ml extract	Angiotensin II (micrograms) per 1mg protein
1 LG	30	207	225	1.08
1 LT	30	390	237	0.61
1 LM	30	322	162	0.50
1 RG	30	25	200	8.00
1 RT	30	724	379	0.52
1 RM	30	465	26	0.06
2 RG	25	72	484	6.73
2 RT	25	130	350	2.69
2 RM	50	190	-	-
3 LG	25	48	265	5.52
3 LT	25	114	400	3.50
3 LM	50	184	275	1.49
3 RG	25	62	317	5.12
3 RT	25	112	317	2.83
3 RM	50	238	234	0.98

FIGURE 19: Dog glomeruli-tubule separation, series II 1970-71 renin distribution

DOG: GLOMERULI-TUBULE SEPARATION. Series II 1970-71

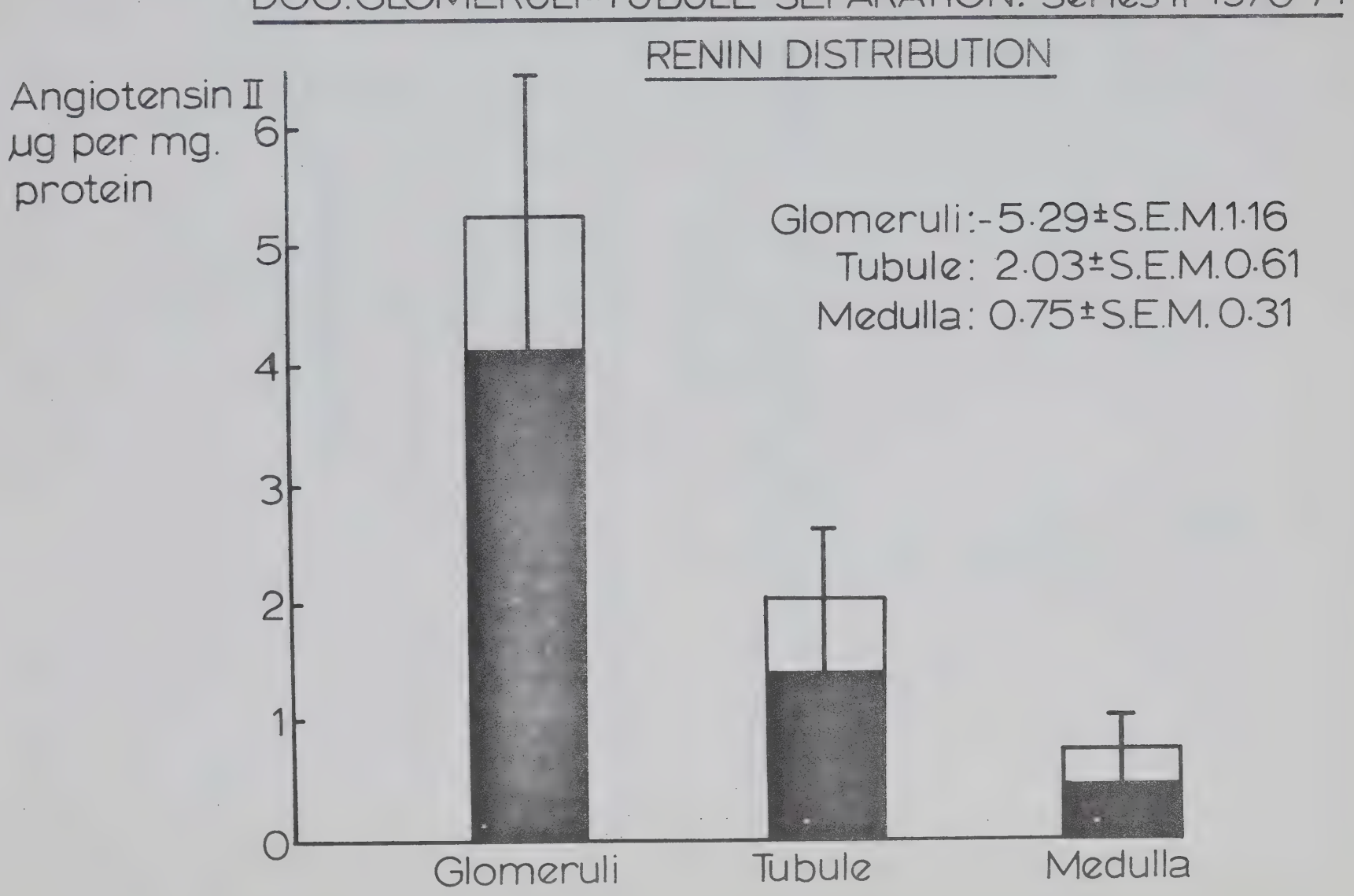


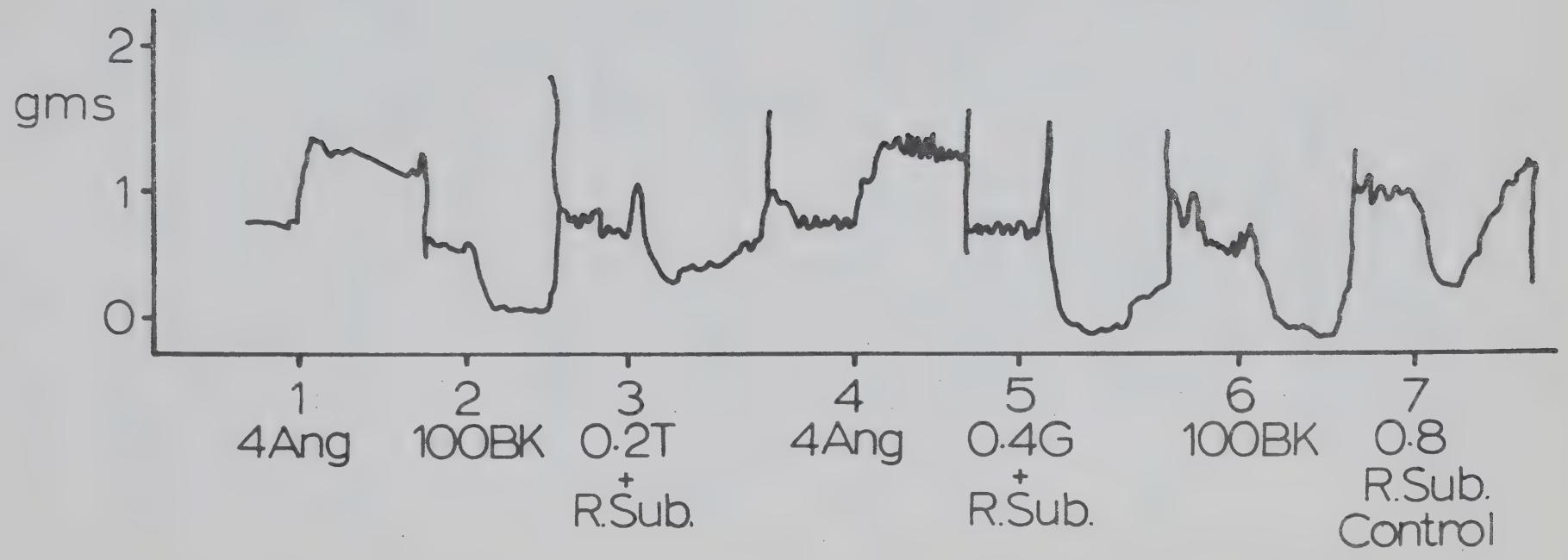
FIGURE 20. The Effects of Angiotensin and Incubates of Dog Renin Extract and Substrate on the Rat Duodenum

Key: - DRS and R. sub refer to dog renin rubitrate
- Ang. angiotensin (nanograms)
- BK. bradykinin (nanograms)
- G. glomerular fraction
- T. tubular fraction

Incubation conditions

1 hour at 37°C. pH 5.2

JANUARY 1971
Rat Duodenum
De Jalons Soln. 31°C
Dog 2. Right Kidney
Renin



followed by relaxation.

Examination of the substrate incubated with isotonic saline as a control, shows that it induced relaxation. This suggests that it contained a significant amount of kinin, and that the intrinsic system of plasma kinin formation was active (plasma kallikrein).

On this basis, it is difficult to interpret the results. However, the initial phase of contraction of the duodenum was consistently found in all the incubates, suggesting that some agent other than kinin was present.

ii. Experiments with carboxypeptidases A and B

The results (FIGURE 21) show that carboxypeptidase A inactivated angiotensin whereas bradykinin was inactivated by carboxypeptidase B. It is seen that the incubates of glomerular and tubular renin extracts contain a substance(s) that is affected by carboxypeptidase A to a greater extent than carboxypeptidase B. This was a consistent finding in all the experiments performed.

The significance of these results is considered in the DISCUSSION.

5. Rat Renal Artery Constriction

A pilot study was performed to evaluate the effects of unilateral renal artery constriction on the concentration of kininogenase in the damaged and contralateral normal kidney. The concentrations of renin were also determined.

Six rats were anaesthetised with an intraperitoneal

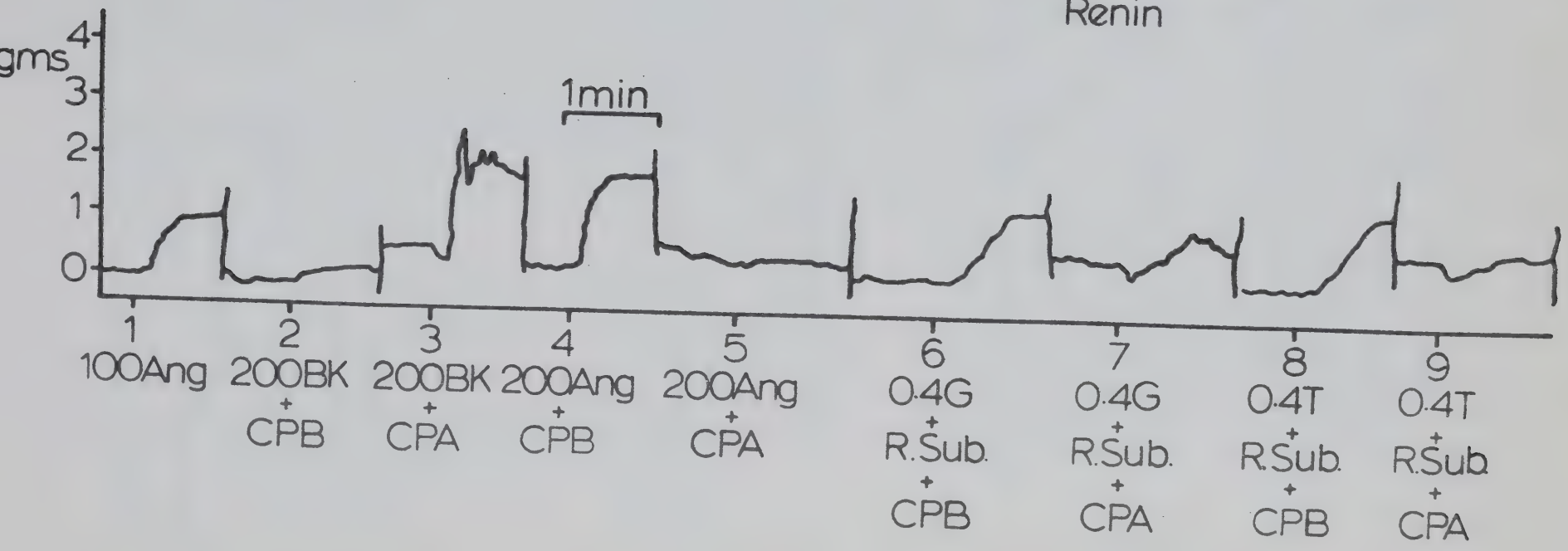
FIGURE 21: The effects of carboxypeptidase A and B on incubates of dog renin extract and substrate

Key: - Ang. angiotensin (nanograms)
- BK. bradykinin (nanograms)
- CPA carboxypeptidase A
- CPB carboxypeptidase B
- G glomerular fraction
- T tubular fraction

Incubation conditions

20 mins at 37°C. pH 7.0

JANUARY 1971
G. pig ileum
Tyrodes Soln. Atr. Mep. 32°C
Dog 2. Right Kidney
Renin



injection of sodium pentobarbital, and an incision made through the abdominal wall to expose the viscera. The left renal artery was exposed and a silver clip with a gap of 0.1 mm. placed over it 0.5 cm. from the hilus of the kidney. The abdominal wall was then repaired and the animal allowed to recover.

After three weeks on a normal diet, the animal was anaesthetised, the systolic blood pressure recorded through an aortic cannula and the kidneys perfused with 0.25 M sucrose, as described under METHODS. The kidneys were then extirpated and divided into halves, which were then extracted for kininogenase and renin.

TABLE 11. Left Renal Artery Constriction, animal and kidney weights

Rat No	Animal weight (gm) Initial	Animal weight (gm) after 3 wks	Kidney wet weight (gm) Left	Kidney wet weight (gm) Right
1	350	420	1.02	1.65
2	370	410	0.74	1.85
3	350	390	0.49	1.52
4	380	465	0.60	2.20
5	380	410	0.52	1.68
6	400	450	0.52	1.90

The animals gained weight during the three weeks following initial surgery and appeared to be healthy on the basis of fur texture and general appearance. When removed, the left kidney was always found to be considerably smaller (TABLE 11) and had a shrunken and pale appearance.

FIGURE 22 shows a normal and an ischaemic kidney, both "in situ" and also following perfusion and extirpation. The silver clip is still present on the renal artery of the shrunken kidney shown in the lower photograph.

The terminal systolic blood pressures and renal kininogenase concentrations are shown in TABLE 12. The final column of the table shows the percentage kininogenase activity of the left as compared to the right kidney (kininogenase activity was expressed as micrograms of bradykinin per gm. wet weight of tissue). It was found that the left kidney contained 45.5 $\pm$ 7.02% kininogenase compared to the right kidney.

The results of renin determinations are shown in TABLE 13. The left kidney was found to contain 90.0 $\pm$ 10.5% renin activity when compared to the right kidney.

The changes in the kininogenase and renin activities are illustrated in FIGURE 23 and the significance of these results considered in the DISCUSSION.

FIGURE 22. Photographs of the Kidneys of a Rat Subjected to Unilateral Renal Artery Constriction

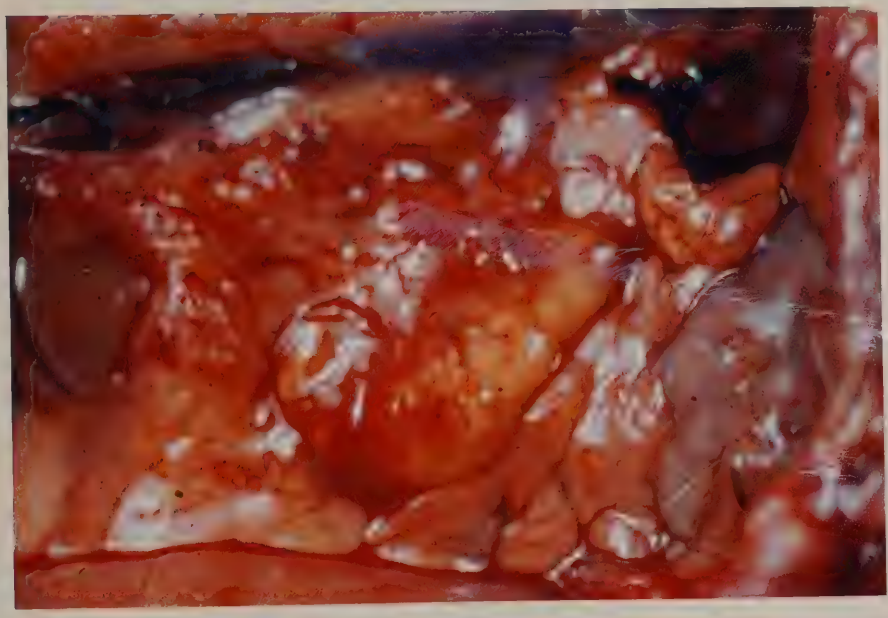


TABLE 12. Rat Renal Artery Constriction 1970

Kininogenase Determinations

0.1 mm. silver clip on left renal artery for 3 weeks

Rat No	Terminal systolic B.P. (mm. Hg.)	ug. bradykinin per gm. dry wt.		ug. bradykinin per gm. wet wt.		% left/right ug. bradykinin per gm. wet wt.
		Left	Right	Left	Right	
1	125	22.2	25.4	2.36	3.54	66.0
2	128	13.5	28.7	1.20	4.36	27.5
3	125	23.9	52.0	1.95	4.59	42.5
4	115	17.4	47.8	1.29	4.72	27.4
5	115	15.2	30.9	1.14	3.67	31.0
6	125	19.2	37.0	1.44	2.37	61.0

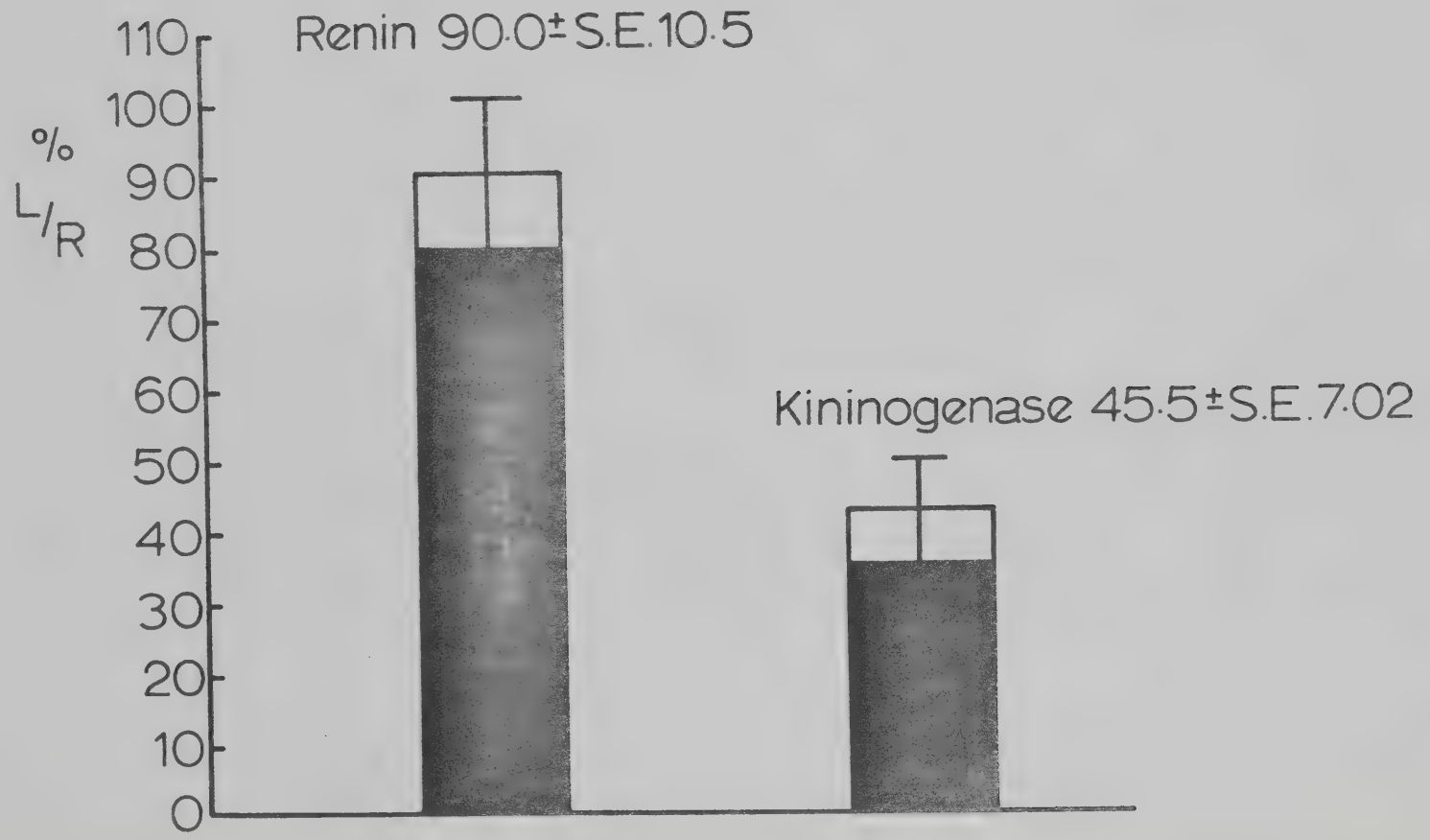
TABLE 13. Rat Renal Artery Constriction 1970

Renin Determinations

Rat No	ug. Angiotensin II per gm. dry wt.		ug Angiotensin II per gm. wet wt.		% Left/right ug angiotensin II per gm. wet wt.
	Left	Right	Left	Right	
1	56.2	54.0	4.8	4.8	100
2	29.0	54.1	3.4	5.0	67
3	61.4	49.6	5.1	5.8	88
4	117.5	97.5	11.4	10.5	109
5	120.0	85.5	9.5	9.6	99
6	57.5	74.8	5.2	8.2	63

FIGURE 23: Rat renal artery constriction.
Changes in kininogenase concentration

RAT RENAL ARTERY CONSTRICTION



DISCUSSION

1. Kininogenases in the Kidney and Urine

The characteristics of kinin-forming enzymes in the kidney and urine have been reviewed in the INTRODUCTION. The properties of rat renal kininogenase have been the subject of several recent studies (Carvalho and Diniz, 1964, 1966; Carvalho, 1970; Nustad, 1969, 1970 a and b) and it has been demonstrated that the enzyme closely resembles rat urinary kallikrein.

In this study, the properties and intra-renal localization of kininogenase were investigated. Kininogenase activity was estimated by the ability of renal extracts to liberate kinins from a plasma protein substrate, however, this process was hindered by the presence of kininase enzymes. A simple technique involving the acidification of renal extracts was found to be an effective inhibitor of kininase activity, while the kinin-forming ability of the extract was apparently unaffected. Utilizing this technique, it was possible to demonstrate kininogenase in the guinea-pig, cat, mouse, rabbit, dog as well as rat kidney. Acidification, as a preparatory procedure for renal kininogenase determination had not been used prior to this study, although its inhibitory effects had been observed (Erdos and Yang, 1966).

The properties of rat renal kininogenase were found to be similar to published findings, with respect to pH

optimum, inhibitor sensitivity and levels of activity. The enzyme was found to be primarily localized within the renal cortex, and the relative activity of the cortex compared to the medulla was found to be 4:1 approximately (FIGURE 10). It was calculated that the cortex contains over 90% of the total kidney kininogenase. Nustad (1970 b) reported similar findings, but with a higher ratio of relative activity (8:1).

The properties of dog renal kininogenase had not been reported prior to this investigation, although the availability of larger tissue samples makes this an attractive species for study. The properties of the enzyme were found to be similar to rat renal kininogenase.

Preliminary experiments suggest that dog urinary and renal kininogenases are similar with respect to pH optima and inhibitor sensitivities, whereas both differ from dog plasma kallikrein. A similar finding in the rat, was cited as evidence to suggest that urinary kallikrein is derived from the kidney and not the plasma (INTRODUCTION).

The effect of renal damage on the excretion of urinary kallikrein also provides evidence to support this theory. The experiments of Croxatto and San Martin (1970 a and b) and Margolius et al., (1972) demonstrated reduced urinary kallikrein concentration in rats subjected to various degrees of renal damage, particularly during renal hypertension. It was found in this study, that unilateral renal artery constriction led to a fall in renal

kininogenase content to 45% as compared to the contralateral normal kidney (FIGURE 23). Further experiments are required (particularly with the inclusion of sham operated controls), before the precise nature of these changes can be established. However, this evidence suggests that the changes in urinary kallikrein excretion observed during renal damage are a reflection of changes in the renal kininogenase content.

The localization of kininogenase in the dog kidney was studied by a technique that isolated glomeruli from the cortex. Three fractions were obtained, glomerular, tubular and medullary, and the kininogenase activity of each determined. The medulla consistently exhibited low levels of activity, whereas the glomeruli and the tubules contained relatively high concentrations (FIGURE 14).

As was demonstrated in the rat, it is seen that kininogenase is primarily found in the cortex. When it is considered that the cortex consists of 85% proximal convoluted tubules (Mattenheimer, 1968), it may be estimated that on the basis of tissue quantity, the largest fraction of the total renal kininogenase is associated with the tubular fraction. In terms of kininogenase concentration per milligram of extractable protein, the glomerular fraction was more active, however, this tissue is a much smaller component of the cortex.

It has been suggested that kininogenase is associated with the proximal convoluted tubule of the rat (INTRODUCTION).

Further evidence in favour of this localization has been obtained by Barton (1970), who found that following unilateral ureteral ligation, a considerable reduction in renal kininogenase activity was observed. After three weeks of ligation, kininogenase levels were reduced to approximately 20% of normal. This procedure causes tubular distension and necrosis, while the glomeruli are relatively unaffected.

Collectively, these findings suggest that in both the rat and dog, renal kininogenase is derived from, and/or secreted by the proximal convoluted tubule. However, the finding of higher concentrations in the glomeruli of the dog kidney cannot be ignored. The significance of kininogenase associated with the glomerulus is discussed in section 2 below.

A series of experiments were carried out to determine the concentration of renin in the three dog renal fractions. Several difficulties were inherent in the techniques utilized. It has been pointed out by Gross (1970) that the bio-assay of angiotensin using the guinea-pig ileum is unreliable, because the preparation develops tachyphylaxis. Also, evidence was presented to show that angiotensin was not clearly identified in the renin incubates. On this basis, the results must be interpreted with caution. It was found that the distribution of renin was very similar to that of kininogenase (FIGURE 19), the highest concentrations being detected in the glomeruli. However, renin was also found to a lesser extent in the

tubular fraction. These results are not in total agreement with the findings of Cook and Pickering (1959), who found renin primarily associated with the glomeruli. However, the localization of renin is still controversial, both glomerular and tubular localizations being possible (Faarup, 1971).

2. The significance of Renal Kininogenase

a) The relationship between kininogenase and renin

Evidence has accumulated to suggest that renin is associated with the juxta-glomerular complex. This complex consists of the afferent and efferent arterioles, lacis cells and the macula densa of the distal tubular segment of the nephron. Granules found in the modified smooth muscle cells of the wall of the afferent arteriole have been found to contain renin (Cook, 1970), and this is considered to be the primary site of synthesis. The subject has been reviewed recently (Page and McCubbin 1968; Cook 1970; Faarup 1971)

It would also seem possible that other components of the juxta-glomerular complex form renin, and the macula densa has received particular attention. It has been shown by Hess and Gross (1959) that renal concentrations of renin are related to the glucose-6-phosphate dehydrogenase activity of the macula densa, suggesting that the latter enzyme is important in the synthesis of renin. Other evidence for the association of renin with tubular structures

is derived from embryological studies, which show that renin is present in the mesonephros and metanephros of the pig foetus before juxtaglomerular granules can be identified by light microscopy (Kaplan and Freidman 1942). The renin content appears to vary with the development of the nephron as estimated by the number of tubules observed in histological sections. Similar studies by Bing and Kazimierczak (1964) and Endes et al., (1965) have shown that this relationship is observed in the kidneys from newborn animals. It has also been demonstrated that renin cannot be extracted from rabbit kidneys in which the proximal tubules are selectively destroyed by sodium tartrate (Freidman and Kaplan, 1943).

The presence of renin and kininogenase in the renal cortex is particularly significant because they both liberate polypeptides from the alpha-2-globulin fraction of the plasma proteins. The similarities between the enzymic release of kinin and angiotensin have been considered by several investigators (Van Arman, 1955; Fasciolo, 1963; Lewis, 1964; Rocha E Silva, 1964, 1970).

Renin releases angiotensin I from the globulin substrate, which is enzymically converted to angiotensin II by a converting enzyme localized in the lungs, renal cortex and other tissues. The pulmonary circulation is considered as the primary site of this conversion. The converting enzyme has been the subject of intensive investigation (Ng and Vane, 1967, 1968; Stanley and Biron,

1969; Barrett and Sambi, 1971; Cushman and Cheung, 1971; Depierre and Roth, 1972; Fitz and Overturf, 1972; Igic et al., 1972; Oparil et al., 1971) and it has been shown that this enzyme is also responsible for the rapid inactivation of bradykinin in the pulmonary circulation.

Although kininogenase and renin release peptides from the same fraction of the plasma proteins, evidence suggests that two distinct substrates are involved (Fasciolo, 1963). This conclusion is based upon depletion studies, where it was found that following depletion of kininogen by kallikrein, plasma can still form angiotensin on incubation with renin. Also, angiotensinogen is destroyed by heating at 37°C at an acidic pH, whereas kininogen remains intact under these circumstances.

In the INTRODUCTION, it was pointed out that a protein-like substance with hypotensive properties commonly contaminated extracts prepared for the bio-assay of the renin-angiotensin system. Pickering and Prinzmetal (1938) wrote that "------ none of our extracts containing renin, however purified, have failed to give depressor responses. Kallikrein may well be present in our preparations of renin, since they behave rather similarly to adsorbants, heat and dialysis, and they are both insoluble in alcohol and concentrated salt solution." This observation was not pursued, however Fasciolo et al., (1958) while investigating the properties of an incubate containing crude renin extract and ox globulin, noted that intravenous administration led to a rise in blood pressure, while a fall was induced

following intra-aortic injection. It is likely that kinin was present in this incubate (Rocha E Silva 1964) and that these findings may be explained by the action of the lung converting enzyme. During intravenous infusion, the pulmonary circulation inactivates bradykinin and converts angiotensin I into angiotensin II. However, during intra-aortic infusion, the bradykinin remains intact and its effect predominates over the less active pressor substance angiotensin I.

Ng (1970) found that crude renin preparations liberated both angiotensin and bradykinin when added to the circulating blood of dogs. It was also found that during experimentally induced renal ischaemia, a condition known to elicit renin release, both peptides were detectable in the arterial blood using the blood-bathed organ technique of Vane. Ng suggests the possibility that renin may release both angiotensin and bradykinin from the plasma proteins. However, until purified preparations of renin become available this suggestion may be considered unlikely due to the possibility of contamination with kininogenase.

Experiments concerning the subcellular localization of renin and kininogenase are not completely resolved. Cook and Pickering (1962) found renin associated with a mitochondrial fraction, which contains lysosomes. The lysosomal localization of renin has since been confirmed (Rouiller and Orci, 1971). Carvalho and Diniz (1966) also find kininogenase associated with this fraction, whereas

Nustad (1970 c) finds kininogenase in the microsomal fraction containing endoplasmic reticulum.

Although the subcellular localization of renin and kininogenase in the kidney remains to be clarified, these substances can be isolated together from granules within the submandibular gland of the white mouse (Werle, 1960). Granules obtained from the homogenized glands of adult male mice contain both substances, and they cannot be separated by centrifugation procedures (Geipert and Erdos, 1971). Electronmicrophotographs of the granules reveal that a heterogeneous population of granules is present. Large granules similar to the kallikrein containing zymogen granules of the pancreas may contain kininogenase, whereas smaller and more amorphous granules similar to those found in the juxtaglomerular complex of the kidney may contain renin. The possibility that both substances are localized within the same granules is also considered possible (Chiang et al., 1968; Geipert and Erdos, 1971).

b) Functional implications

The role of kininogenase in the kidney and urine is unknown. The evidence presented in this study suggests that urinary kininogenase is secreted by the kidney. This situation is analogous to that found in the salivary glands and pancreas.

Concerning the role of renal kininogenase, one may speculate that this enzyme is involved in the antihypertensive action of normal renal tissue. However, the half-life of

kinins in the circulation is less than one second, over 80% being inactivated during one passage through the lung (APPENDIX). If the kininogenase-kinin system is involved in an antihypertensive function, it is unlikely that this effect could be mediated by the systemic release of kinins.

The possibility that intra-renal kinin release may be of functional importance is more attractive. Intra-arterial infusions of bradykinin and kallidin lead to arteriolar vasodilatation and an increased kidney volume. Renal blood flow is increased and a diuresis is observed which is associated with substantial increases in sodium and chloride secretion, the observed changes are thought to be secondary to an increase in glomerular filtration (Webster and Gilmore, 1964; Jacobson, 1970). Thus, intra-renal kinin release may be involved in the regulation and re-distribution of renal blood flow. This would be most effectively achieved if kininogenase was localized in or near to the glomerulus. Kinin released in this situation would affect both glomerular filtration and the intra-renal distribution of flow. The experiments reported in this study provide evidence that kininogenase is localized in the glomerular tissues. A local renal action of kininogenase does not exclude the possibility of an antihypertensive function, because the regulation of renal cortical blood flow may modify the response of the renin-angiotensin system. Cortical ischaemia is the major stimulus for renin secretion,

however, it is clear that the intra-renal action of kininogenase would tend to suppress the activity of this system. Hence an antihypertensive action is possible, purely on a local renal basis.

These speculations offer impetus to the further investigation of renal kininogenase. Although this enzyme is found in high concentrations in the glomerular tissues, it was argued in Section 1, above, that the largest fraction of total renal kininogenase is associated with the tubular elements of the cortex. This conclusion is supported by other studies, however, the functional implications of tubular kininogenase are more difficult to evaluate. One may speculate that intra-tubular kinin release may influence the excretory functions of the tubule, or may influence the renal vasculature by re-absorption into the renal interstitial tissues.

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APPENDIX

The Kallikrein-kinin System

1. Nomenclature

The following nomenclature has been adopted in this study, following the recommendations of the "committee on nomenclature for hypotensive peptides", held on October 27th 1965, in Florence, Italy (Webster 1970 b).

Kininogenase. Any enzyme that liberates a kinin from an inactive protein substrate. Thus the kallikreins, trypsin, pepsin, snake venoms, bacterial proteinases and other proteolytic enzymes are included.

Kallikrein (EC.3.4.4.21). A kallikrein is defined as an endogenous enzyme which rapidly and specifically liberates a kinin from the kininogens of plasma. The inactive enzyme precursor of kallikrein is known as prekallikrein. Each kallikrein is identified by the species and tissue of origin, for example; rat urinary kallikrein.

Kininase. A general name to describe enzymes which inactivate kinins.

Kininogen. A protein, usually derived from plasma, that will release a kinin by the action of proteolytic enzymes.

Kinins. A kinin, is a general name indicating a hypotensive polypeptide that contracts most isolated smooth muscle preparations but relaxes the rat duodenum.

Kinins increase vascular permeability, produce pain when applied to an exposed blister base on human skin, and cause bronchoconstriction in the guinea-pig. Bradykinin and kallidin (Lys-bradykinin) are the most intensively investigated peptides in this group, although the term kinin applies to any peptide either synthetic or naturally occurring, which is structurally related to bradykinin.

2. Kininogenases, with Particular Reference to Kallikreins

The subject has been extensively reviewed in recent years (Kellerman and Graham 1968; Schachter 1969; Erdos 1970; Rocha E Silva 1970), hence a brief summary will be provided here.

Kallikreins are defined as "endogenous enzymes which rapidly and specifically liberate a kinin from the kininogen substrate of plasma". Kininogenases on the other hand, differ from the kallikreins in that they also show a more generalised proteolytic activity towards other natural substrates. All crude kallikrein preparations show non-specific activity, however, during purification this diminishes, whereas kinin-releasing activity, pharmacological potency and rate of hydrolysis of synthetic esters all increase (Werle and Trautschold 1963). In order to classify an enzyme as a kallikrein, its substrate specificities must be established, which requires the preparation of purified extracts. This has only been performed in a few cases and hence, a large number of

enzymes, some of which may eventually be shown to be kallikreins are classified as kininogenases.

A variety of enzymes release kinins from plasma kininogen substrate (Prado 1970), these include trypsin, pepsin, carboxypeptidase A, bacterial proteases, papain, ficin and the snake venoms. Snake venoms are particularly interesting because the kininogenase present is in part responsible for their toxic effects. Studies with A.halys blomhoffii suggest that venom kininogenases show many of the properties of mammalian kallikreins, and may be considered to be the salivary kallikrein of this species (Suzuki and Iwanaga 1970).

Kininogenase activity has been detected in many tissues, secretions and exudates including; cerebro-spinal fluid, bronchial mucosa, lymph glands and spleen, brain and peripheral nerves, sweat gland secretions, carcinoid tumour and its metastases, lacrimal secretions, perfusates of the glandular elements of the tongue and acid treated homogenates of epithelial cells from the intestinal mucosa.

a) Chemical properties of the kallikreins

Kallikreins are found in an active form in the urine, saliva, salivary glands and pancreatic juice. Inactive prekallikreins are found in the plasma, pancreas and kidney.

Kallikreins derived from different sources, although similar in their pharmacological properties, show distinct chemical differences. They are not only species specific,

but also vary from tissue to tissue within the same animal. The amino acid compositions of several kallikreins have been reported and they have been shown to be glycoproteins, however, little is known about their amino acid sequences and tertiary configurations.

Fritz et al., (1967) detected multiple forms of kallikrein in the hog pancreas. The sialic acid content varied, but even after its removal by neuraminidase, two distinct forms could still be identified, with molecular weights of 33,000 and 29,000. Nustad (1970 a) estimated the molecular weights of rat renal and urinary kallikreins as 38,500 while Pierce (1970) suggestedamolecular weight of 30,000 for human salivary kallikrein.

Prekallikreins have been isolated from the pancreas, and found to have similar molecular weights to the kallikreins to which they are converted. This conversion appears to be proteolytic and Schachter(1969) speculates that the "inactivating fragment"must be smaller than any of the natural inhibitors commonly associated with kallikreins. Activation may well involve a relatively minor molecular alteration, perhaps of the allosteric type.

Plasma kallikreins, are much larger molecules with molecular weights varying from 97,000 to 163,000 depending upon the source (Pierce 1970). This may explain the differences observed between plasma and glandular kallikreins with respect to inhibitors. Also, plasma kallikreins show a more generalized proteolytic activity than is the case

with other kallikreins.

b) Species specificity

Webster (1970) reviews the subject and quotes many examples of species specificity. It is clear that this specificity lies not only with the kallikreins, but also the substrate. Hog, horse, guinea-pig and rat glandular kallikreins release the maximum amount of kinin from kininogens of the same species. Horse urinary and salivary kallikreins are the only known kallikreins that will release kinin from horse plasma, however, horse urinary kallikrein will release small quantities of kinin from the plasma of other mammalian species. The reason for the specificity of kallikrein-substrate complexes is unknown, although it probably lies in the conformation of the "active centres" of the molecules.

c) Sensitivity to inhibitors

Frey and Kraut (1928) demonstrated that the contamination of urine with blood led to the inactivation of urinary kallikrein and hence they postulated the presence of an inhibitory substance in plasma. Subsequently, Kraut et al., (1930) found very large quantities of a kallikrein inhibitor in the bovine pancreas.

Since these early discoveries, a variety of substances from plant and animal tissues, as well as various chemical inhibitors, have been found to inhibit a wide variety of proteolytic enzymes including the kininogenases and the kallikreins. The subject is reviewed by Vogel and Werle (1970).

Naturally occurring inhibitors may be divided into two groups, high molecular weight substances such as those derived from the plasma, egg white, urine and various plants, and low molecular weight substances such as the polyvalent bovine trypsin inhibitor (Trasylol). The inhibitors appear to create enzymically inactive complexes, such that the activity towards natural and synthetic substrates is suppressed. The formation of the complex is pH dependent and is usually optimal in the pH range 7 to 9. The complexes have been found to exist in a definite molar ratio of enzyme to inhibitor (1:1), and complete recovery of enzyme activity usually occurs following its dissolution.

All serums appear to inhibit a wide variety of proteolytic enzymes and many inhibitors have been isolated. Some are polyvalent and affect many proteases, whereas others are highly specific. Each kininogenase is blocked by more than one serum inhibitor, and Vogel and Werle (1970) suggest that local minimal activation of kininogenases is instantly blocked by these substances under physiological circumstances.

Several inhibitors are of particular interest, as they were utilized in this investigation. Soya Bean Trypsin Inhibitor (SBTI) and Ovomucoid Trypsin Inhibitor (OMI) inhibit plasma kallikrein, whereas the activity of glandular and urinary kallikreins is relatively unaffected (Werle and Maier, 1952). This provides a method of differentiating between plasma and glandular kallikreins.

The polyvalent bovine inhibitor (Trasylol) has aroused much interest because of its wide spectrum of inhibitory effects. It affects the hypotensive, esterase and kininogenase activities of most kallikreins, with the exception of dog urinary kallikrein (Frey, Kraut and Werle, 1968). It is found in the lymph nodes, spleen, parotid glands, lungs, liver and pancreas of the ruminants, particularly cattle.

Bovine inhibitor was studied by Kunitz and Northrop as a trypsin inhibitor, and by Frey, Kraut and Werle as a kallikrein inhibitor. Its amino acid sequence has been determined and the kinetics of the inhibitor-enzyme complex are the subject of investigation at the present time (Huber et al., 1971; Vincent et al., 1971).

It is thus possible to differentiate between the various kallikreins and kininogenases by the use of selective inhibitors. However, it is also clear that the availability of an inhibitor to block the kininogenase activity of an extract will depend on the pH, temperature and concentration of contaminants. Hence, inhibitor studies are ideally performed on purified enzyme preparations.

3. Natural and Synthetic Substrates

It has been established that there are two or more kininogen substrates in the plasma protein complex. The significance of this is far from clear, however, there is evidence to suggest that certain kallikreins have

specific and differing substrate affinities.

Van Arman (1955) found that kininogen was localized in the Cohn Fraction IV-4 (subfraction IV-6) of plasma. It was found to be an alpha-2-globulin sedimenting between 33 and 45% saturation with ammonium sulphate. It was named "pseudoglobulin by Rocha E Silva et al., (1949).

Biochemical studies provide clear evidence for the existence of two or more kininogens in the plasma of many mammals. Habermann and Rosenbusch (1962) described the isolation of two acidic glycoproteins from bovine plasma using gel filtration, a high molecular weight kininogen (HMW) and a low molecular weight kininogen (LMW). Pierce and Webster (1966) isolated two LMW kininogens from human plasma with molecular weights of approximately 50,000, both being glycoproteins containing hexosamine, hexose and neuraminic acid. The subject is reviewed by Habermann (1970).

The existence of several kininogens is also suggested by the studies of Eisen and Vogt on glass activated plasma, where it was found that plasma kallikrein, and a second kininogenase specifically activated by glass contact, consumed separate substrates (Eisen and Vogt, 1970).

Jacobsen (1966) demonstrated two separate kininogens in the plasma and lymph of several species, both having different kininogenase affinities. Gautvik et al., (1968) showed that one of these substrates (substrate I) was exclusively consumed by plasma kallikrein, whereas substrate

II was consumed by glandular kallikreins. Hilton (1970) was studying the relative depletion of these two substrates during functional vasodilatation of the cat submandibular gland. He found that the level of substrate II in the venous blood from the gland was significantly reduced, whereas substrate I was unaffected. This evidence was thought to indicate that during functional vasodilatation, substrate II was consumed by salivary gland kallikrein, the kinin released being responsible for the blood flow changes observed.

Kallikreins, although specific with respect to natural substrates, share the ability to hydrolyse synthetic esters with many other proteolytic enzymes. Trypsin, chymotrypsin, kininogenases and kallikreins hydrolyse esters such as - N-benzoyl-L-arginine-ethylester (BAEE), benzoyl-l-arginine-methylester (BAME), toluolsulphonyl-l -argininemethylester (TAME) and N-acetyl-l-tyrosine-ethylester (ATEE),

Esterase activity can be used as a means of identification of proteolytic enzymes because these enzymes cleave the substrates at different rates. The advantage of using synthetic substrates, is that hydrolysis products can be measured very accurately using chemical techniques.

The use of synthetic substrates is reviewed by Trautschold (1970), Moriya et al., (1971, and Beaven et al., (1970). Trautschold et al., (1969) have described the

determination of kallikrein with the Technicon Autoanalyser using synthetic substrates.

4. The Chemical and Biological Properties of the Kinins

Bradykinin and kallidin, are the two kinins of physiological significance in mammalian systems, however, various other kinins have been isolated from venoms, amphibian skin and avian plasma (Schachter, 1970; Pisano, 1970). It has been stated that plasma kallikrein liberates bradykinin from kininogen, whereas glandular kallikreins release kallidin (Pierce, 1970), however, this is considered to be an oversimplification (Schachter, 1969).

Elliot et al., (1960) purified bradykinin from trypsin-activated extracts of ox plasma. Sequence analysis initially revealed that bradykinin was an octapeptide, however, Boissonnas et al., (1960 a) synthesized this structure and found it biologically inactive, Subsequently, a nonapeptide was synthesized with biological and pharmacological properties identical to bradykinin (Boissonnas et al., 1960 b).

Pierce and Webster (1961) obtained kallidin from incubates of urinary kallikrein with acid-treated plasma, and the peptide was synthesized by Nicolaides and Dewald (1961).

The structure of the two peptides is shown below:

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

bradykinin

Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

lys-bradykinin (kallidin)

Kinins exert many pharmacological effects, which may be summarized with respect to the site of action as follows:

a) Systemic and local circulation

b) Capillary permeability

c) White blood cell migration

d) Sensory nerve endings

e) Smooth muscle

a) Systemic and local cardiovascular actions

Bradykinin lowers the systemic arterial pressure of all species studied, and the effect has been attributed to a fall in total peripheral resistance due to arteriolar vasodilatation. Intra-arterial injection of bradykinin leads to a decreased resistance in the coronary, renal, whole fore-limb, hind-limb, forearm, calf, hand, skin, and the superior mesenteric, ileal, stomach, bronchial, internal carotid and salivary gland vascular beds (Haddy et al., 1970). Arteriolar vasodilatation has been directly observed by Baez and Orkin (1963) and Zweifach (1964, 1966).

In terms of relative potency, bradykinin is approximately ten times more active than acetylcholine as a hypotensive agent (on a molar basis), for example, it has been shown that bradykinin is less potent on a weight-to-weight basis in producing vasodilatation of the cat hind limb, however, it is more potent when considered on a molar basis (Elliot et al., 1960).

The results of experiments utilizing intravenous injection should be interpreted with caution because of the

very short half-life of bradykinin in the circulation (0.27 minutes, 0.32 minutes for kallidin) (McCarthy et al., 1965). Ferreira and Vane (1967 a, b and c) showed that 80% of the peptide was inactivated by a single passage through the pulmonary circulation, and the presence of kininases in the blood and other tissues contributes to this rapid destruction. Thus, after intravenous injection, only a fraction of the peptide reaches the peripheral vascular bed. Higher doses are hence required, and the possibility of indirect reflex responses is introduced.

An increased cardiac output is commonly observed following the intravenous injection of bradykinin. The increase in stroke frequency and volume appears to be due to increased venous return, increased myocardial strength and indirect reflex effects. Bradykinin in physiological concentrations has little effect upon the frequency and strength of the isolated heart (Antonio and Rocha E Silva, 1962; Nakano, 1965), and there is abundant evidence that cardiovascular compensatory reflexes are involved.

Frequently, following intravenous injection, the hypotensive effect is not maintained and a pressor overshoot is observed (Croxatto and Belmar, 1961; Feldberg and Lewis, 1964). The pressor overshoot is inhibited by reserpine, phenoxybenzamine, chlorpromazine and hexamethonium (Rocha E Silva et al., 1960; Lang and Pearson, 1968) and also by adrenalectomy.

It is particularly significant that the hypotensive

effect of bradykinin is converted to a pressor response by pentolinium (Croxatto and Belmar, 1961; Montague et al., 1963) and guanethidine (Miele and DeNatele, 1967). The significance of this finding was considered in the DISCUSSION.

The compensatory reflexes elicited by intravenous injection of bradykinin may arise through several pathways. Hypotension leads to sympatheticoadrenal responses due to baroreceptor activity, also there is evidence to suggest that kinins directly stimulate adrenomedullary secretion of catecholamines (Feldberg and Lewis, 1964) and release peripheral catecholamine stores (Montgomery and Kroger, 1967).

b) Capillary permeability

Increased capillary permeability following the injection of bradykinin has been demonstrated with pontamine sky blue (Bhoola et al., 1960), Evans blue (Zweifach, 1966) and Trypan blue (Rocha E Silva 1940). Bradykinin induces the passage of fluochromated plasma proteins into the mesenteric interstitial fluid (Witte et al., 1961) and Sturmer (1966) detected an increase in the protein concentration and flow of lymph from the perfused dog hind limb.

Rocha E Silva (1970) compares the activity of several potent agents, pointing out that bradykinin is consistently the most potent substance affecting capillary permeability. Local administration of bradykinin kallidin or kallikrein leads to the rapid efflux of water from

systemic vascular beds, and Haddy et al., (1970) discussed the mode of action. It is thought that arteriolar dilatation associated with either little or an actual increase in venous resistance, leads to an increased transmural pressure in the capillary bed. The increased pressure forces plasma colloids into the tissues, and as a consequence, a translocation of fluid occurs.

c) White blood cell migration

Menkin (1936) prepared a material from inflammatory exudates that produced vascular permeability and leukocyte migration, the active principle was named "leukotaxin". Subsequently it was demonstrated that tryptic digests of serum contained a substance with similar actions (Menkin, 1940). The possibility that bradykinin was responsible was examined by Lewis (1963), who demonstrated leukocyte accumulation around the site of intradermal injection of bradykinin in the guinea-pig and rabbit. Leukocyte migration was also observed following the topical application of bradykinin to the rabbit mesentery.

d) Sensory nerve endings, pain production

The pain-producing action of the plasma kinins was first described by Armstrong et al., (1952, 1953 a and b) comparing the pain induced by a variety of agents injected intradermally or applied to the base of a cantharidin blister.

Acetylcholine produced an immediate needle-like pain of rapid onset and decline, with little after-pain or

burning sensation. 5-hydroxytryptamine and bradykinin produced a burning pain of delayed onset, slow increase to peak and slow decline. The pain producing actions of histamine required relatively high concentrations (5×10^{-3}M) and are probably of minimal significance. 5-hydroxytryptamine and bradykinin were much more potent, the effective concentrations for threshold being 10^{-6}M for 5-hydroxytryptamine and 10^{-7} to 10^{-8}M for bradykinin.

Plasma was found to be inactive, however, after storage in glass it produced a typical delayed pain. The name "pain producing substance" was given to the active principle, which was shown by Andrade and Rocha E Silva (1956) to have all the pharmacological properties of plasma - generated bradykinin. The pain producing polypeptide was found in aspirates from cantharadin blister fluid, heat burn blister fluid, insect bite blister fluid, rheumatoid arthritis fluid, inflammatory pleural effusions and hydrocele fluid.

The generation of "pain-producing substance" from plasma can be inhibited by soya bean trypsin inhibitor (SBTI) and hexadimethrine bromide (or protamine sulphate) which inhibits the activation of Hagemann factor. The similarity between pain-producing substance and the kinins is sufficient to indicate their possible role in the generation of pain associated with inflammation.

e) Smooth muscle

The action of kinin hormones on various smooth

muscle preparations has been reviewed recently (Walaszek, 1970; Rocha E Silva, 1970; Trautschold, 1970). Bradykinin and kallidin are qualitatively very similar in their actions, displaying only quantitative differences.

The rat uterus is the most sensitive preparation available responding to 10^{-8} to 10^{-10}gm, ten to a thousand times smaller than that required for the guinea-pig ileum (10^{-7} to 10^{-9}gm) (Rocha E Silva, 1970). The response of the rat uterus to bradykinin is similar to oxytocin, however, the latter has a negligible effect on the guinea-pig ileum and rat duodenum.

The action of bradykinin on the guinea-pig ileum takes much longer than histamine and acetylcholine, and the recovery is also prolonged, taking up to a minute following washing with Tyrodes solution. The addition of antihistamines and atropine to the bathing fluid reduces the spontaneous activity of the preparation, as well as blocking the effects of histamine and acetylcholine, which may be present as contaminants in tissue extracts.

Relaxation of the rat duodenum is very characteristic of the kinin hormones, the threshold concentration being 0.1 ng/ml. Under similar conditions angiotensin produces a rise in tone of the muscle, a mixture of bradykinin and angiotensin producing the algebraic sum of their individual actions.

A variety of other smooth muscle preparations have been utilized for detecting kinin peptides, including hen

rectal caecum (relaxation), rat fundus strip, rabbit large intestine, cat jejunum, spiral strips from sheep and ox carotid artery and human intestine. These and other preparations are discussed in the reviews mentioned above.

5. The Distribution and Actions of Kininases

Kininases are enzymes that inactivate kinins by enzymic degradation. They are widely distributed throughout the tissues of all animals investigated, and have also been found in plants, bacteria and molds. The subject is reviewed by Erdos and Yang (1970).

The rapid inactivation of kinins in the blood is in part due to the presence of kininases in the red blood cells, leukocytes, thrombocytes and the plasma. Together with the naturally occurring protease inhibitors of plasma (Section 2, c above) they provide a protective mechanism against the hypotensive effects of intravascular kinin formation.

Carboxypeptidase N was the first kininase to be identified in the plasma (Erdos, 1961). It cleaves the carboxyl terminal arginine of bradykinin as well as attacking this same site in the kallidin molecule. A second kininase (kininase II) has also been isolated from the plasma of most species of mammals including man. The enzyme cleaves the Pro^{7}-Phe^{8} link and is very similar to the kininase found in the kidney and lung (DISCUSSION).

Plasma kininases are irreversibly inactivated by

acidification or heat treatment. Acidification to pH 2.0 or heating at 56°C for five minutes are effective inhibitors, and because of this, crude kininogen preparations are commonly prepared by heating and/or acidification (METHODS).

Evidence has been presented to suggest the existence of two kininogenase-kininogen systems in the plasma (Eisen and Vogt, 1970), the presence of two kininases might be of functional importance in this respect.

Kininases have been found in the urine, bile, colostrum, ascites, synovial fluid and saliva. Observations on urine and lymph suggest that both carboxypeptidase N and kininase II are present.

Homogenates from many tissues contain kininase, however, the pancreas, kidney, and lung are particularly active. Kininase activity in the kidney is very high and exceeds that of plasma. Three kininases have been identified in the swine kidney, and the enzymes have been localized to the microsomal fraction of homogenates by Erdos and Yang (1966). Renal kininases are active at neutral pH but are inactivated in acidic conditions below pH 5.0. This latter finding is particularly significant since acidification was used for kininase inactivation in this study (RESULTS).

6. The Physiological significance of the kallikrein-kinin system

The quotation of Sir Henry Dale (1933) (INTRODUCTION) is pertinent when considering the physiological role of the kallikrein-kinin system. Although much evidence is available to suggest that the potent actions of the kinin hormones are involved in physiological regulation, definite proof is lacking.

The kallikrein-kinin system consists of two components, the glandular kallikreins and the plasma kallikreins. Hilton and Lewis (Hilton, 1970 for review) have postulated that the glandular kallikreins are mediators of functional vasodilatation in glandular tissues. The evidence for this view is based upon the finding of kininogenase activity in perfusates of the submandibular gland, pancreas, tongue and human skin (during sweating) following stimuli that produce functional vasodilatation. It has been recently demonstrated that submandibular kallikrein consumes a specific kininogen separate to that consumed by plasma kallikrein. It was demonstrated that this kininogen was partially depleted in the venous effluent of active submandibular glands, providing further evidence of kinin release during glandular activity.

Schachter and his colleagues do not support this theory (Schachter, 1971, for review). In the guinea-pig, active kininogenases are only found in the submandibular and prostate gland and hence kininogenase cannot be

involved in the hyperaemia of the other glands of this species. Also, the submandibular kininogenase of the guinea-pig cannot release kinin from guinea-pig plasma. More direct evidence against the role of kallikrein in the functional hyperaemia of glandular tissues has been provided. Depletion of glandular kallikrein of the cat submandibular gland by ductal ligation does not affect the vasodilatory response, nor does perfusion of the gland with a kininogen-free perfusate. Desensitisation of the gland to bradykinin by the intra-arterial injection of high doses of the substance were also found to be ineffective in blocking the vasodilatation, as was the injection of carboxypeptidase B, a potent inactivator of the kinins.

A wide variety of experimental conditions have been shown to induce the generation of kinins by the plasma kallikrein system. The complex nature of the enzymes in the plasma makes the study of plasma kallikrein difficult, however, it would seem that the enzyme is activated by tissue damage and immune reactions (Eisen and Vogt, 1970 for review). A relationship between plasma kallikrein and the enzymes involved in hemostasis and fibrinolysis has been established, but is poorly understood, however, such a relationship may prove to be of physiological importance in the regulation of the inflammatory process. The properties of kinins, as reviewed in Section 3 above, suggest that they are capable of mediating many of the events characteristic of tissue injury, including vasodilatation, capillary permeability, white cell migration and pain.

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